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Assessment of macrophage and granulocyte cell-enhanced phagocytosis of *Mycobacterium tuberculosis* by novel monoclonal antibodies

By

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Declaration

I hereby declare that the work on which this dissertation is based, is original, and has been documented as such. This thesis has not been submitted before for any degree purposes at this or any other University. Any statements or inferences made which do not originate from me have been accurately referenced.

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Signature of Candidate

Dedication

This thesis is dedicated to my parents, Shey Henry N and Ali Miriam M Epse Shey.

"Those who do wickedly against the covenant he shall corrupt with flattery; but the people who know their God shall be strong and carry out great exploits."

Daniel 11:12

Therefore,

"I can do all things through CHRIST who strengthens me."

Philippians 4:13

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List of Abbreviations

ATCC	American type culture collection
CFU	Colony-forming unit
CGM	Complete growth medium
cMtb	Clinical susceptible *M. tuberculosis* patient strain
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GU	Growth unit
HDT	Host-directed therapy
HL60/HL-60	Human promyelocytic leukaemia cells (granulocyte cell line)
IFN-γ	Interferon gamma
IgG/IgG1	Immunoglobulin gamma/immunoglobulin gamma 1
IL	Interleukin
kg	Kilogram
MABs/mAbs	Monoclonal antibodies
MDR-TB	Multi drug resistant tuberculosis
MGIT	Mycobacterium growth incubation tube
MNLs	Mononuclear leukocytes
SMEG/*M. smegmatis*	*Mycobacterium smegmatis*
MTB/*M. tuberculosis*	*Mycobacterium tuberculosis*
mg	Milligram
mL	Millilitre
mM	Millimolar
ng	Nanogram
OADC	Oleic acid, agarose, dextrose, catalase
OD	Optical density
OP	Opsonophagocytic
OPKA	Opsonophagocytic killing activity
pg	Picogram
RT	Room temperature
rpm	Revolutions per minute
TB	Tuberculosis
TNF-α	Tumour necrosis factor-alpha

U-937	Human myelomonocytic leukaemia cells (macrophage cell line)
XDR-TB	Extensively drug resistant tuberculosis
µg	Microgram
µL	Microliter
%	Percentage
ºC	Degree Celsius

Publications and Conference Presentations

Publications

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1. **University of Pretoria: Faculty of Health Sciences Research Symposium, 2017**
   Venue: University of Pretoria, Prinshof Campus
   Authors: Bong-Akee Shey, Kudzai B Nyazema, Nontuthuko E Maningi, Clara J Sei, P Bernard Fourie
   Title: Demonstrating specific binding of novel mouse IgG1 monoclonal antibodies to *Mycobacterium tuberculosis* towards enhancing autophagy
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2. **Fifth South African National Tuberculosis Conference, 2018**
   Venue: Durban, South Africa
   Authors: Bong-Akee Shey, Clara J Sei, Nontuthuko E Maningi, P Bernard Fourie
   Title: Demonstrating specific binding of novel mouse IgG1 monoclonal antibodies to *Mycobacterium tuberculosis* towards enhancing autophagy
   Presentation type: Oral
3. **Twenty-ninth European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 2019**
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   Authors: Bong-Akee Shey, Clara J Sei, Kudzai B Nyazema, Theresa M Rossouw, Nontuthuko E Maningi, Gerald W Fischer, P Bernard Fourie
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Assessment of macrophage and granulocyte cell-enhanced phagocytosis of *Mycobacterium tuberculosis* by novel monoclonal antibodies

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SUMMARY

Mycobacterium tuberculosis (*M. tuberculosis* or MTB) is a complex bacterium that causes the highly infectious disease, tuberculosis (TB), which is generally transmitted to a healthy individual after the inhalation of droplet nuclei, sneezed or coughed out by an infected individual. Phagocytes such as alveolar macrophages, monocytes, neutrophils and dendritic cells, residing in the lungs, are employed by the host as the first line of defence against *M. tuberculosis* infection. These phagocytes also help in recruiting components of secondary (adaptive) immunity such as antibody-producing cells, which contribute to host protection by targeted binding to *M. tuberculosis* antigenic receptors, thereby preventing *M. tuberculosis* attachment to other host cells and tissues. This action also aids in opsonizing *M. tuberculosis* for easy elimination by these phagocytes.

Considerable work has been done in the area of TB therapeutics in order to improve treatment outcomes for *M. tuberculosis* infection. Host-directed therapeutic (HDT) strategies are currently being investigated as a potential avenue to facilitate this outcome. One of such HDTs involving

monoclonal antibodies (mAbs or MABs), demonstrates potential, as mAbs of the immunoglobulin gamma (IgG) class have been shown *in vitro*, to possess immunotherapeutic properties against *M. tuberculosis,* with reactivity to epitopes present on both live and killed *M. tuberculosis*. The development of an HDT based on mAbs would require high mycobacterial cell-wall binding activity by the mAb, and phagocyte enhancement, for *M. tuberculosis* elimination.

This study demonstrates the ability of a selected novel IgG1 mouse monoclonal antibody, JG7 III D3 I F9 (JG7), to enhance the phagocytic elimination (opsonophagocytosis) of susceptible *M. tuberculosis* strains at both their mid-logarithmic and stationary growth phases, by human U-937 macrophage and HL-60 granulocyte cell lines which are involved in intracellular killing of bacteria, and to assess the cytokine response to this process. In principle, the involvement of macrophages, which form part of the host's innate immune response, and monoclonal antibodies, which are activated by components of the humoral arm of the adaptive immune response, will imply components of both innate and adaptive immunity are at play in mounting a stronger defence against these *M. tuberculosis* strains.

The activity of novel mAbs towards binding to various *M. tuberculosis* strains in order to determine their efficacy for enhancing the phagocytic and antimicrobial activities of U-937 macrophage and HL-60 granulocyte cell lines have been investigated before, acting as a stimulus for embarking on the current study directed at mAb JG7. Killed forms of clinically susceptible, multidrug resistant and extensively drug resistant *M. tuberculosis* strains were used in indirect enzyme linked immunosorbent assays (ELISAs), and JG7 concentrations between 0.156 µg/mL and 10 µg/mL were assessed while live forms of clinically susceptible *M. tuberculosis* strains were used in live bacteria ELISAs, and JG7 concentrations between 0.25 µg/mL and 25 µg/mL were assessed. An increase in JG7 concentration led to an increase in binding activity for both live and killed *M. tuberculosis*.

Investigations undertaken in this study, aimed to determine if the novel mAb JG7 might enhance the ability of human U-937 macrophage and HL-60 granulocyte cell lines to phagocytose *M. tuberculosis,* are also reported. Two susceptible *M. tuberculosis* strains (H37Ra and cMtb) were used at both their mid-logarithmic and stationary growth phases in *in vitro* opsonophagocytic (OP) assays, and JG7 concentrations between 0.25 µg/mL and 100 µg/mL were assessed. The OP assays were performed independently on both susceptible *M. tuberculosis* strains at both

bacterial growth phases, using both U-937 and HL-60 cell lines. In addition to JG7 concentration and bacterial growth phase, factors such as bacterial dilution alongside other secondary factors, were assessed. Opsonophagocytic killing activity (OPKA) was determined by comparing the bacterial colony-forming units (CFUs) of the samples containing JG7 with those of the control with no JG7, on Middlebrook 7H10 or 7H11 agar. Decreases in bacterial CFUs were observed with the samples compared to the controls, for both susceptible *M. tuberculosis* strains used, and with both human cell lines.

Further statistical analyses (linear prediction models) were performed on the data obtained from the OP assays. According to this, OPKA was demonstrated by low bacterial CFUs relative to baseline (controls with no mAb) even at the lowest JG7 concentration of 0.25 µg/mL. An increase in mAb concentration led to an increase in OPKA, with the highest activity demonstrated at 1 µg/mL and 10 µg/mL in U-937 and HL-60 cells, respectively. OPKA was higher at the lower bacterial dilution of 1:100 compared to the higher bacterial dilution of 1:1000 for both H37Ra and cMtb in both human cell lines, with statistically significant differences in OPKA observed between dilutions 1:100 and 1:1000, for H37Ra with the HL-60 cell line and for cMtb with the U-937 cell line.

The last part of this study was aimed at determining whether there is a change in the cytokine response with the addition of JG7 during the opsonophagocytic process. One *M. tuberculosis* strain was used at mid-logarithmic phase, and a JG7 concentration of 25 µg/mL was assessed. Multiplex suspension array assays, investigating seven cytokines, were performed independently, using both human granulocyte and mononuclear cells isolated from four individuals. Four sub-groups were investigated; unexposed human cells as a negative control (Sub-group 1), human cells exposed to *M. tuberculosis* (Sub-group 2), human cells exposed to both *M. tuberculosis* and mAb JG7 (Sub-group 3), and human cells exposed to lipopolysaccharide as a positive control (Sub-group 4). A cytokine response was determined by comparing the median fluorescent intensity (observed concentration) of each sample, with that of the experimental standards. The observed concentrations for all seven cytokines fell within the normal range of the experimental standards with all four sub-groups.

Descriptive statistics using medians and interquartile ranges showed that, with the granulocytes, levels of several pro-inflammatory cytokines – interleukin (IL)-1β, tumour necrosis factor (TNF)-α and IL-8 (sub-group 3 only) – were significantly higher in sub-groups 2 and 3 when

compared to the negative control, while not quite achieving levels expressed by the positive control. With MNL cells, higher levels of all the cytokines, except IL-12p70 (which was only higher in sub-group 2), were observed in sub-groups 2 and 3 when compared with the negative control. Higher levels of IL-1β, interferon (IFN)-γ, and TNF-α, and lower levels of IL-10, were observed in sub-groups 2 and 3 when compared to the positive control. There was a large, but non-significant, increase in IL-8 expression by the granulocytes exposed to the mAb. In contrast, the mAb-exposed MNL cells expressed significantly lower levels of IL-12p70, together with a large, but non-significant, increase in the expression of IL-10.

In summary, according to results from both ELISA techniques, mAb JG7 affinity for each bacterial strain increased with an increase in the mAb concentration. The indirect ELISAs showed JG7 binding to all *M. tuberculosis* strains used, though at different affinities. Optimal binding occurred at the 1:100 bacterial dilution, and at the highest JG7 concentration of 10 µg/mL. For the live bacterial ELISAs, higher JG7 concentrations had to be used to achieve better binding and optimal binding also occurred at the 1:100 bacterial dilution and at the highest JG7 concentration of 25 µg/mL. For the *in vitro* OP assays performed, higher JG7 concentrations had to be used in order to determine maximum killing effect. The assays showed JG7-enhanced phagocytosis to all *M. tuberculosis* strains used, though at different levels. There was a relative increase in OPKA with increase in mAb concentration, at each bacterial dilution assessed (1:100 and 1:1000), and this increase was demonstrated mostly with the cMtb strain by the U-937 macrophage cell line. For the multiplex suspension array assays, the JG7 concentration of 25 µg/mL was selected, since it was used in both binding and OP assays. The assays showed that the mAb induced a possible pro-inflammatory effect, driven by IL-8, in neutrophils and a significant anti-inflammatory response in MNL cells, as demonstrated by the significant decrease in pro-inflammatory IL-12p70 and increase in anti-inflammatory IL-10.

These investigations showed some degree of consistency from the binding to the killing assays, since specific JG7 concentrations were shown to bind and enhance killing of *M. tuberculosis* (2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 25 µg/mL). However, more studies would help confirm these findings, and using this design of mAb-enhanced phagocytosis and cytokine response measurements in animal studies, is the next logical step. Finally, investigating the effect of this novel mAb in animals when paired with standard anti-TB chemotherapy is an essential step towards establishing a potential HDT.

Chapter 1

1 Introduction

1.1 Background

On the 24th of March 1882, Robert Koch announced the discovery of *Mycobacterium tuberculosis* (*M. tuberculosis*) (Koch, 1882; Sakula, 1979). This event rapidly led to a broader understanding of the pathology of the disease, tuberculosis (TB), caused by this organism. *M. tuberculosis* is a complex bacterium that causes TB, a highly infectious disease, which is generally transmitted to a healthy individual after the inhalation of droplet nuclei (particles of approximately 1 - 3 microns in diameter) sneezed or coughed out by an infected individual (Ahmad, 2011). Infection begins with the entry and passage of *M. tuberculosis* particles through mucosal surfaces in the host. Their small sizes help the bacilli evade the sweeping action of the cilia in the bronchi of the lungs (Ahmad, 2011). The bacilli further travel to the terminal alveoli and end up in antigen-presenting cells or phagocytes which consist predominantly of alveolar macrophages, monocytes, neutrophils and dendritic cells residing in the lungs (Kolloli and Subbian, 2017). This phagocyte response is intrinsic and almost immediate (the first line of defence by the host) (Schafer *et al*., 2009). Subsequently, a second adaptive component of the host immune response consisting of T-lymphocytes (cell-mediated response) and B-lymphocytes (humoral response), develops (Ahmad, 2011). There is, however, a complex interplay resulting in a series of events between host and pathogen which ultimately determines the overall outcome of infection (Upadhyay *et al*., 2018).

Although a lot of insight into *M. tuberculosis* pathology has been gained since Koch's discovery of the organism more than a century ago, the organism still causes an enormous worldwide burden of disease; an average of 10 million people developed the disease in 2018 alone (WHO, 2019). One of its primary successes depends on evading the antimicrobial action of phagocytic macrophages, despite the fact that macrophages present an acidic and degradative environment containing several carriage routes for phagocytosis which lead to the production of reactive oxygen and nitrogen species that induce the expression of pro-inflammatory cytokines that enhance the host's response (Köster *et al*., 2018). Unfortunately, *M. tuberculosis* can render the environment in the macrophage tolerant by manipulating host innate immune responses and cell

death pathways to its benefit (Köster *et al*., 2018). The organism not only hinders phagosome-lysosome fusion (in the macrophage) which is essential for bacterial clearance, but also cripples the phagosomal trafficking mechanism responsible for lysosomal degradation (Sugaya *et al.*, 2011; Upadhyay *et al*., 2018). This failure in macrophage function results in about a third of the overall population infected progressing to active disease (WHO, 2018). Fortunately, most individuals infected are able to control the infection in a clinical state of latency due to their natural immunity, although, the risk of reactivation disease is a tangible threat in these individuals (Barry *et al.*, 2009).

While the cell-mediated immune system is essential for the host to defend itself against TB, there is evidence suggesting that specific antibodies referred to as monoclonal antibodies (mAbs or MABs), which form part of the humoral immune system, also limit the spread of infection via mucosal immunity. These humoral responses expressed during active TB disease have, however, not been fully characterized (Nunes-Alves *et al*., 2014; Jacobs *et al.*, 2016). Monoclonal antibody therapy if widely explored is expected to contribute to global TB control programmes, because it has the potential to increase the efficiency of anti-TB treatment (Zumla *et al.*, 2013; Kolloli and Subbian, 2017).

1.2 Rationale and problem statement

Chemotherapy is the most common means of treating TB infection, although the success rate for eliminating active bacteria is only about 97% and persisters are not fully cleared, thereby increasing the chances of recurrent disease (Kolloli and Subbian, 2017). Also, many patients with TB, including those who are compliant with treatment schedules, nevertheless relapse after the completion of therapy and relapse cases to effective regimens have increased from 5% of the overall population (including clinical trial settings) undergoing treatment, to more than 10% in recent times (Colangeli *et al*., 2018; Romanowski *et al*., 2018). Optimizing chemotherapy is desirable and one possible approach towards such an intervention is immune-enhancement by means of host-directed therapy (HDT) or immunotherapy. The use of mAbs in the course of active disease, during latency, or after presumed cure, might enhance clearance of tubercle bacilli and prevent persistent infection and recurrent TB disease. By enhancing the elimination of *M. tuberculosis*, mAbs might aid in diminishing the risk of treatment failure and relapse.

1.3 Aims and objectives

The aim of this study was to demonstrate, by an *in vitro* model, the ability of a selected novel IgG1 mouse mAb JG7 III D3 I F9 (hereafter referred to as JG7) to aid in the phagocytic elimination (opsonophagocytosis) of *M. tuberculosis* by human monocyte and granulocyte cell lines. The hypothesis was that mAb JG7 would enhance the opsonophagocytic elimination of various susceptible *M. tuberculosis* strains in human hybridoma U-937 monocyte (become macrophages upon differentiation) and HL-60 granulocyte cells and that there would be an alteration in cytokine production in response to the opsonophagocytic process. Three major techniques were applied to this effect; *in vitro* opsonophagocytic killing assays, flow cytometry assays, and multiplex suspension array assays.

The experimental objectives were pursued by: (1) Using cell culture techniques in order to passage and differentiate the human macrophage and granulocyte cell lines, in preparation for the opsonophagocytic killing assays. (2) Demonstrating that JG7 enhances the phagocytic/opsonic elimination action of both the human macrophage cell line U-937 and human granulocyte cell line HL-60 against live susceptible *M. tuberculosis* (H37Ra ATCC 25177 and clinical susceptible TB) *in vitro,* by opsonophagocytic killing assays. (3) Isolating human granulocyte and mononuclear cells from healthy donors, in preparation for the multiplex suspension array assays. (4) Assessing the purity and viability of these human granulocyte and mononuclear cells, by flow cytometry assays. (5) Demonstrating, by multiplex suspension array assays that, the human cell: *M. tuberculosis:* mAb interaction that occurs during opsonophagocytosis elicits a cytokine response, and (6) assessing the cytokine response profile during this interaction for both granulocyte and mononuclear cells.

1.4 Thesis structure

C**hapter 2** presents an overview of recent advances made in the area of TB diagnostics, vaccines and therapeutics, as well as the current challenges. The chapter discusses current HDTs, with emphasis on the role of mAbs and the processes they employ in order to enhance the elimination of *M. tuberculosis*. In **Chapter 3**, findings are presented on mAb-binding to various *M. tuberculosis* (susceptible, multi-drug resistant and extensively drug resistant) strains, as well as preliminary data on opsonophagocytosis of live susceptible *M. tuberculosis* by the human U-937

macrophage cell line. **Chapter 4** expands on the opsonophagocytic killing of both laboratory strain H37Ra (ATCC 25177) and a clinical susceptible TB (patient) strain at two independent phases of bacterial growth (mid-logarithmic and stationary phases), by both human U-937 macrophages and HL-60 granulocytes. **Chapter 5** discusses the cytokine response upon human granulocyte and mononuclear cell interaction with *M. tuberculosis* (H37Rv ATCC 26518) and compares this response with that of a three-way interaction between each human cell line, *M. tuberculosis* H37Rv, and novel mAb JG7 during opsonophagocytosis. **Chapter 6** concludes the thesis by presenting key findings obtained in the course of this investigation, as well as possible research avenues that could be explored as a means of widening the scope for future investigations.

1.5 References

Ahmad S (2011) Pathogenesis, immunology, and diagnosis of latent *Mycobacterium tuberculosis* infection. *Clinical and Developmental Immunology* doi: 10.1155/2011/814943.

Barry CE, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, Schnappinger D, Wilkinson RJ, Young D (2009) The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nature Reviews Microbiology* **7**: 845–55.

Colangeli R, Jedrey H, Kim S, Connell R, Ma S, Chippada Venkata DU, Chakravorty S, Gupta A, Sizemore EE, Diem L, Sherman RD, Okwera A, Dietze R, Boom HW, Johnson LJ, MacKenzie RW, Alland D for the DMID 01-009/Tuberculosis Trials Consortium Study 22 Teams (2018) Bacterial Factors That Predict Relapse after Tuberculosis Therapy. *New England Journal of Medicine* **379**: 823–33.

Jacobs JA, Mongkolsapaya J, Screaton RG, McShane H, Wilkinson JR (2016) Antibodies and tuberculosis. *Tuberculosis* **101**: 102–113.

Koch R (1882) "Die aetiologie der tuberculose. *Berliner Klinische Wochenschrift*" **19**: 221–30.

Kolloli A and Subbian S (2017) Host-Directed Therapeutic Strategies for Tuberculosis. *Frontiers in Medicine* **4**: 171.

Köster S, Upadhyay S, Philips JA (2018) Why macrophages cannot LAP up TB. *Autophagy* **14**(3): 552–554.

Nunes-Alves C, Booty MG, Carpenter SM, Jayaraman P, Rothchild AC, Behar SM (2014) In search of a new paradigm for protective immunity to TB. *Nature Reviews Microbiology* **12**: 289–99.

Romanowski K, Balshaw FR, Benedetti A, Campbell RJ, Menzies D, Khan AF, Johnston CJ (2018) Predicting tuberculosis relapse in patients treated with the standard 6-month regimen: an individual patient data meta-analysis. *BMJ Journals* doi: http://dx.doi.org/10.1136/thoraxjnl-2017-211120.

Sakula A (1979) Robert Koch: founder of the science of bacteriology and discoverer of the tubercle bacillus. *British Journal of Diseases of the Chest* **73**: 389–94.

Schafer G, Jacobs M, Wilkinson RJ, Brown GD (2009) Non-opsonic recognition of *Mycobacterium tuberculosis* by phagocytes. *Innate Immunity* **1**(3): 231–43.

Sugaya K, Seto S, Tsujimura K, Koide Y (2011) Mobility of late endosomal and lysosomal markers on phagosomes analyzed by fluorescence recovery after photobleaching. *Biochemical and Biophysical Research Communications* **410**: 371–375.

Upadhyay S, Mittal E, Philips JA (2018) Tuberculosis and the art of macrophage manipulation. *Pathogens and Disease* **76**(4).

World Health Organization (2018) *Global tuberculosis report 2018* Geneva. Licence: CC BY-NC-SA 3.0 IGO.

World Health Organization (2019) *Global tuberculosis report 2019* Geneva. Licence: CC BY-NC-SA 3.0 IGO.

Zumla A, Nahid P, Cole ST (2013) Advances in the development of new tuberculosis drugs and treatment regimens. *Nature Reviews Drug Discovery* **12**: 388–404.

Chapter 2

2 Diagnosis, treatment and prevention of tuberculosis: Review of current status

2.1 Introduction

Tuberculosis (TB) is the most prevalent bacterial infectious disease worldwide. Caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*), it accounts for around 4000 deaths daily (WHO, 2018). Although the World Health Organization (WHO) has established goals towards a 95% reduction in TB deaths and a 90% reduction in TB incidence by 2035, *M. tuberculosis* does not allow for a single mechanism of intervention to be considered effective, given the bacterium's complex ability to exacerbate disease or evade capture (WHO, 2018). A better understanding of the biology of *M. tuberculosis,* with a view towards development of improved assays, vaccines and therapeutics, is still greatly needed if the global epidemic is to be halted.

2.2 Advances and challenges in the diagnosis of tuberculosis

Major progress has been made from the era of presumptive diagnosis (that deals mostly with signs and symptoms of TB) to definitive diagnosis whereby the presence of the organism is objectively confirmed. The desired objective in this area is a timely and efficient diagnosis, so that patients get treated quickly in order to limit the spread of infection; this is one of the sustainable developmental goals pursued by the WHO (WHO, 2018). An accurate and safe test is dependent on the identification of an appropriate biomarker that is either host- or pathogen-specific, and the identification of these biomarkers in turn requires the proper biological specimen (bio-specimen) from patients, as well as the right technique or approach in order to characterize TB in these patients (Ota *et al.*, 2014; Swanepoel *et al.*, 2015). Appropriate methods need to be applied, depending on the type of specimen being investigated, as discussed below.

2.2.1 Biological specimens used in TB diagnosis

Several bio-specimens are currently in use for TB diagnosis since the organism can thrive almost anywhere in the human host, and the use of unconventional specimens such as breath and saliva

have even been attempted. However, sensitivity is crucial when it comes to the type of specimen produced (Phillips *et al*., 2010; Ota *et al*., 2014). The following biological specimens are currently being used as diagnostic tools in TB research, and some of these specimens will be discussed alongside the markers they produce as well as the most common techniques used in the assessment of these markers.

Skin: A lot of information can be gained from the skin and this is evident in one of the oldest techniques used as a diagnostic in TB research, the tuberculin skin test (TST). The TST measures the degree of skin infiltration as an expression of a delayed-type hypersensitivity reaction (occurs within 48 and 72 hours), after the intradermal injection of purified protein derivative (PPD) which is a group of antigenic proteins that are not unique to, but are present in *M. tuberculosis* (Pai *et al*., 2014; Goletti *et al*., 2016). The TST gives an indication of an encounter with TB, whether in the past or recently. A response is therefore produced for both active and latent disease. This test unfortunately suffers from low specificity since, not only can it not differentiate between active and latent infection, but infection by any member of the mycobacterium genus produces a response, as can the Bacille Calmètte-Guérin (BCG) vaccine (Doherty *et al*., 2007; Swanepoel *et al*., 2015).

Sputum: Sputum is the most commonly collected diagnostic sample and sputum smear microscopy, which measures the presence of *M. tuberculosis* bacilli, is the most commonly used diagnostic test in patients with pulmonary TB in high burden and low-income countries (Davies *et al.*, 2013). Although sputum smear microscopy (or microscopy as it is commonly called), is widely available and highly specific, it requires high quality specimens and has a low sensitivity, thereby missing the diagnosis in over one third of patients seeking care, since many patients with active disease, including immunocompromised patients and children, often do not present *M. tuberculosis* in their sputum (Lagrange *et al*., 2012; Goletti *et al*., 2016). Furthermore, several clinic visits are required for standard sputum collection and TB confirmation although it has been claimed that submitting one sputum sample provides similar results to submitting more than one (Walzl *et al*., 2011; Davies *et al*., 2013). Sputum culture on Löwenstein-Jensen (LJ) agar is still the gold standard for confirmatory TB diagnosis in some settings, even though it provides results after a considerable delay (3 - 4 weeks). Furthermore, it can neither differentiate between susceptible and drug resistant *M. tuberculosis* strains, nor is it reliable in monitoring treatment efficacy since sputum conversion does not necessarily translate to a reduction in colony-forming

units (CFUs) on agar (Goletti *et al*., 2016). The introduction of the BACTEC system, which uses the mycobacterial growth incubation tube (MGIT) and Middlebrook 7H9 broth, enhanced the diagnostic algorithm because, in addition to measuring sputum conversion by time-to-positivity, it also gives some preliminary indication of resistance profiles through drug susceptibility testing. However, some might argue that the turn-around time or culture time-to-positivity of approximately 2 weeks, is still lengthy.

Blood: Intravenously derived blood and different blood components such as plasma and serum are used in TB diagnostics. Although blood collection is an invasive and painful sampling method, there are a host of immunological biomarkers in blood, which are useful in identifying *M. tuberculosis* (Wang *et al*., 2018). The most common are cytokines and antibodies as seen in whole blood, and protein biomarkers and T-cells as seen in serum (Adekambi *et al*., 2012). Blood is not used in routine diagnosis, especially not in resource limited settings, and only biochemical, immunological, or genotypic tests are currently being carried out using this bio-specimen.

Urine: Urine collection is one of the safest and least painful sampling procedures, and urine contains a range of excretion markers. But despite the amount of research that has gone into urine as a bio-specimen, to date, only one urine biomarker-based test has been endorsed by the WHO; the urine lipoarabinomannan (LAM) test (WHO, 2011; Yerlikaya *et al*., 2017). The LAM test measures the pathogen-specific biomarker LAM, which is a cell wall component of *M. tuberculosis* (Yerlikaya *et al*., 2017). Unfortunately, fluctuations occur in urine, thereby affecting the stability of the biomarkers involved, which is why this test has been considered unreliable due to its poor sensitivity (Minion *et al*., 2011). Nevertheless, it has been found to be useful for TB diagnosis in human immunodeficiency virus (HIV)-infected patients with low cluster of differentiation 4 (CD4) counts (Goletti *et al.*, 2016).

Stool: Stool collection is also a relatively safe and less painful sampling method that was introduced for the diagnosis of TB recently. Stool has been attempted in children with pulmonary TB since *M. tuberculosis* from swallowed sputum is thought to present in stool (Walters *et al*., 2017). There are currently no phenotypic tests for stool, and only molecular testing is performed with extracted *M. tuberculosis* deoxyribonucleic acid (DNA) from these samples (Walters *et al*., 2017; Rahman *et al*., 2018).

Fine-needle aspirates: Fine-needle aspirates such as lymph node aspirates, are specimens that are relatively easy to collect; unfortunately, the sampling method is rarely used in hospital settings (Fanny *et al*., 2012). Individuals who are unable to produce sputum, e.g. infants, are a suitable target for fine-needle aspiration. The fluid obtained can be used for several types of investigation, including microscopy, culture, and molecular tests (Fanny *et al*., 2012).

Other biological fluids: These are unusual and invasive samples that are mostly collected when *M. tuberculosis* causes disease other than pulmonary TB (Goletti *et al.*, 2016). Cerebrospinal fluid (CSF) is commonly used for microscopy and GeneXpert techniques, when there is suspicion of TB meningitis. Biomarkers, such as tumour necrosis factor alpha (TNF-α), have also been associated with disease severity in samples such as broncho-alveolar lavage (BAL), and T-cells isolated from CSF or pleural effusions have been investigated (Doherty *et al*., 2007; Walzl *et al*., 2011).

2.2.2 Molecular and immunological techniques used in TB diagnosis and research

In resource limited settings, confirmatory tests such as culture, which measures either colony conversion (time-to-positivity) or the number of CFUs (colony enumeration) and serve as endpoints in diagnosis or treatment, are time consuming and limiting. For example, measuring time-to-positivity is only practical in liquid cultures like MGIT. Therefore, molecular assays are required for greater sensitivity and specificity. Unfortunately, these techniques are costly and most of the high-burden areas that need them are relatively poor.

There has been significant progress in research and innovation with regards to TB diagnosis. A technique that lacks sensitivity or specificity cannot truly identify a biomarker of interest. This is the case with the expensive chest X-ray technique that went obsolete for a while but is now gradually re-entering the field of diagnostics. Johnson *et al.*, (2009) found an increase in the number of relapse cases, as well as extensive lung damage, in patients without chest X-ray cavities whose sputum culture tests were negative, 2 months after the initiation of treatment (Johnson *et al*., 2009; Walzl *et al.*, 2011). Better techniques are therefore required for diagnosis and a number of these are currently being applied in the field. Most of these techniques are biochemical, immunological and molecular assays which are more sensitive and provide more information on the organism as well as the host. Unfortunately, these assays are more expensive and hence not feasible for use in routine laboratories. They are therefore mostly used only for

research purposes. Some of the assays currently in use alongside the biomarkers and bio-specimens they investigate include:

Polymerase chain reactions: The standard polymerase chain reaction (PCR) technique relies on the detection of insertion sequence 6110 (IS6110) which is found in *M. tuberculosis* DNA where it is present in multiple copies (Thabet *et al*., 2014; Löpez-Hernandez *et al*., 2016). PCR has been used on sputum extracts to determine the presence of *M. tuberculosis* ribonucleic acid (RNA) (biomarker used to identify active TB through viable bacteria) or *M. tuberculosis* DNA (shows encounter with TB, whether active or not). Modifications of the PCR technique, such as multiplex assays (real time PCR), are also in use. Pinhata and colleagues have used the multiplex platform to confirm the *M. tuberculosis* complex protein 64 (MPT64) antigen in *M. tuberculosis*-infected patients (Pinhata *et al*., 2015). PCR is the basic principle for most molecular assays used in TB diagnostics.

GeneXpert-based tests: The Xpert MTB/RIF and Xpert MTB/RIF Ultra assays are PCR-based diagnostic tools currently in use. These are cartridge-based automatic nucleic acid amplification assays that, in addition to identifying *M. tuberculosis* DNA through the detection of the IS6110 and IS1081 genes (the additional IS1081 gene for Xpert Ultra) in unprocessed sputum and stool samples, also detect the mutations of the *rpo*B gene that are responsible for more than 95% of known rifampicin resistance mutations among *M. tuberculosis* strains, found within the rifampicin resistance-determining region (RRDR) (Boehme *et al*., 2011; Walters *et al*., 2017; Rahman *et al*., 2018; Makhado *et al*., 2018). Since their introduction as complementary tests to the MGIT, there has been a lot of hype about their having high sensitivities with both pulmonary and extrapulmonary TB, with their only limitation being the high cost (Boehme *et al*., 2011). However, their ability to serve as useful tools in monitoring treatment response is limited, since they pick up both live and dead *M. tuberculosis* DNA (Goletti *et al*., 2016). Furthermore, these tests neither detect rifampicin resistance associated with *rpo*B mutations found outside of the RRDR, nor do they determine drug resistance due to isoniazid mono-resistance, thereby leading to misdiagnosis and incorrect treatment regimens that further promote resistance (Makhado *et al*., 2018).

Line probe assays: The line probe assay (LPA) isolates *M. tuberculosis* DNA or RNA directly from raw sputum or from culture, and reverse hybridizes it onto a nitrocellulose membrane containing immobilized probes with different resistance mutations (Luetkemeyer *et al*., 2014).

These membranes or strips are interpreted using a template. LPA is a molecular method used in the diagnosis of TB and in the detection of both rifampicin and isoniazid resistance (Singh *et al*., 2017). The GenoType MTBDRplus version 1.0 identifies rifampin and isoniazid resistance by detecting the most common mutations of the *rpo*B, the *kat*G and *inh*A genes, respectively (Luetkemeyer *et al*., 2014). The newer version of LPA, the GenoType MTBDRplus version 2.0, has an added advantage; it can be used on both scanty positive and smear-negative sputum samples (Singh *et al*., 2017).

Interferon gamma release assays: The interferon gamma release assays (IGRAs) are a group of assays that measure the release of interferon gamma (IFN-γ) by antigens such as the early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are specific to the *M. tuberculosis* complex, with the exception of BCG sub-strains (Doherty *et al*., 2007; Pai *et al*., 2014). These antigens are encoded by genes located within the region of difference 1 (RD1) locus of the *M. tuberculosis* genome (Pai *et al*., 2014). IGRAs such as the QuantiFERON TB Gold In-tube and enzyme linked immunospot assay (T-SPOT.TB), measure IFN-γ expression from T-cell response since IFN-γ is thought to be a good biomarker (Doherty *et al*., 2007). However, IFN-γ levels may simply be a measure of inflammatory status (Walzl *et al*., 2011). In addition to their relatively low sensitivity, IGRAs do not differentiate between active and latent infection since they reflect both current bacterial load as well as the T-cell memory responses generated due to past infection, thereby making interpretation of IFN-γ results difficult in isolation (Doherty *et al*., 2007; Walzl *et al*., 2011).

Chromatography: Chromatography is based on the separation of molecules due to differences in their structure and/or composition, when a sample mixture applied to a stable surface (stationary phase), interacts with that stationary support with the help of a mobile phase, leading to its separation into different components (Kupiec, 2004). Generally, molecules that form stronger interactions with the stationary phase are slower in their movement through the column than components with weaker interactions (Kupiec, 2004). The difference in the types of chromatography is brought about by the difference in stationary phases such as: immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography) (Kupiec, 2004). Both liquid and gas chromatography have been evaluated for the diagnosis of TB. The organic compounds in a breath study by Phillips *et al*., (2010) were analysed using gas chromatography and mass

spectrometry. High performance liquid chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in solution (Conte Junior *et al*., 2015). It has been used for the rapid identification of *Mycobacterium* species isolated from culture, based on the presence of different mycolic acids, with *M. bovis* and *M. tuberculosis* sharing similar signatures (Conte Junior *et al*., 2015).

Mass spectrometry: Mass spectrometry (MS) is used for both the identification and characterization of molecules based on fragmentation and ionization, and the determination of their mass-to-charge ratio (López-Hernandez *et al*., 2016). MS uses molecular weight profiling as a means of measuring *M. tuberculosis* protein biomarkers in blood. This is demonstrated in a study by Hare and colleagues, who found 68 proteins to be associated with immune function, lysosomal/endosomal maturation, vesicular formation, nucleosome proteins, and antigen processing, all of which seemed to be indicators of the degree of TB infection (Hare *et al.*, 2015). The matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) is based on the same principle as MS. It is also used in the identification of bacteria based on their protein profiles, by comparing the mass spectra separated according to their TOF in samples such as urine and blood (Neuschlova *et al*., 2017). MALDI-TOF has the added advantage of producing strong solvents capable of lysing bacteria without any pre-treatment (Neuschlova *et al*., 2017). Wang and colleagues demonstrated by MALDI-TOF-MS, 2-dimensional gel electrophoresis, and bioinformatics techniques, that a combination of three urinary biomarker proteins; mannose-binding lectin 2 (MBL2), inter-alpha-trypsin inhibitor heavy chain 4 precursor (ITIH4-35k), and micro ribonucleic acid 625 (miRNA-625-3p), performed best for the diagnosis of pulmonary TB (Wang *et al*., 2018). Nevertheless, the technique is rarely used due to the complexity of the proteome since it consists of numerous analytes, undergoes post-translational modifications, and forms many complexes, presenting its own challenges in biomarker discovery (Hare *et al*., 2015). Furthermore, analytical challenges such as algorithms for protein identification, the difference in spectra readings, the different fragmentation methods, as well as the accuracy of instruments used, also come into play (Hare *et al*., 2015).

Gel electrophoresis: Studies have demonstrated the use of urine proteins as potential diagnostic biomarkers in determining TB disease occurrence and progression, by techniques such as gel electrophoresis, a method used to separate, identify and purify nucleic acids (Lu *et al.*, 2011;

Young *et al*., 2014; Wang *et al.*, 2018). Electrophoresis is the separation of charged DNA molecules in an applied electric field since electricity causes these molecules to migrate towards the electrode of opposite charge, thus separating the DNA based on its mass and charge (Reddy and Raju, 2012). The movement of DNA molecules in the field depends on several factors including: charge/mass ratio, shape, temperature, porosity and viscosity of the gel through which the DNA molecules migrate (Reddy and Raju, 2012). Young *et al.*, (2014) used a combination of 1-dimensional gel electrophoresis, HPLC and MS to identify 20 human proteins as significant discriminators of TB disease. Pulsed field gel electrophoresis (PFGE) is another gel electrophoresis technique, whereby the whole *M. tuberculosis* genome is digested by special restriction enzymes such as XbaI, AseI, DraI, SpeI, and passed through an alternating electric field, not supportable or moveable by common gel electrophoresis techniques (Pooideh *et al*., 2015). It is used to determine the genetic relatedness or diversity of *M. tuberculosis* strains and to detect contamination sources among other things. This is a more efficient way to control the spread of TB especially because, using the entire genome of *M. tuberculosis* instead of short repeat elements, provides more information (Pooideh *et al*., 2015).

Lateral flow assays: The lateral flow assay (LFA) is based on the principle that a liquid sample containing an analyte of interest moves by capillary action, through various zones on a polymeric strip containing molecules that can interact with the analyte (Koczula and Gallotta, 2016). The test strip contains antibodies that are specific to the target analyte and are conjugated to coloured or fluorescent particles for better stability and detection (Koczula and Gallotta, 2016). It is a safe and easy technique to use, and relatively small sample volumes are required for analysis. This assay has been used to investigate *M. tuberculosis* markers in saliva and blood (Ota *et al*., 2014; Manga *et al.*, 2016). The commonly used MPT64 *M. tuberculosis* antigen test, is based on this principle (Jacobs *et al.*, 2016). The rapid 123 TB-XT HEMA EXPRESS test is also an LFA that has been developed for the detection of immunoglobulin gamma (IgG) antibodies in blood and serum, against proteins produced by members of the *M. tuberculosis* complex (Manga *et al.*, 2016).

Flow cytometry: Flow cytometry measures the degree and direction in which light scatters as it passes through a particle. As fluorescently labelled cells are passed in single file in front of a laser, they are excited by the laser to emit light and are thereby identified, counted and sorted based on the scattering of this light at various wavelengths (Fuhrmann *et al*., 2008). This

technique has been used to measure cluster of differentiation (CD)4+ and (CD)27+ T-cell populations in adults from a low TB endemic country in order to differentiate between various stages of TB infection, and has also been used to assess the expression of cytokines such as IL-2 and TNF-α, in BCG-vaccinated individuals (Fuhrmann *et al*., 2008; Petruccioli *et al*., 2015).

Restriction fragment length polymorphism: Restriction fragment length polymorphism (RFLP) is a technique that measures the difference in homologous DNA sequences, detected by the presence of DNA fragments of varying lengths after the digestion of said DNA with specific restriction endonucleases (Pooideh *et al*., 2015). The IS6110-RFLP depends on the copy number of the IS6110 sequence in *M. tuberculosis* isolates and is effective for isolates with six IS6110 copies or more in their genomes (Pooideh *et al*., 2015). It is used as a typing technique in assessing the genetic diversity and clonal similarities between strains in *M. tuberculosis* isolates, as this provides useful information for controlling the spread of TB (Pooideh *et al*., 2015).

Spoligotyping: Spacer-oligo-typing commonly referred to as spoligotyping, is a hybridization assay which detects any variability in the direct repeat (DR) region of *M. tuberculosis* DNA (Ei *et al*., 2016). The DR region is made up of multiple copies of a conserved 36 base pair sequence separated by various unique spacer sequences (Ei *et al*., 2016). The entire DR locus is amplified by PCR and the PCR products are hybridized onto a membrane containing 43 spacer oligonucleotides (Ei *et al*., 2016). Spoligotyping is used for the identification and classification of *M. tuberculosis* strains into lineages. Furthermore, it can identify *M. tuberculosis* strains with lower IS6110 copy numbers than RFLP (Ei *et al*., 2016). It is also one of the most frequently used PCR-based molecular typing methods in TB research (Ei *et al*., 2016).

MIRU-VNTR: The *M. tuberculosis* genome consists of 40 to 100 base pairs of repetitive sequences called “mycobacterial interspersed repetitive units” (MIRU), found throughout said genome (Ei *et al*., 2016). These MIRUs have different characteristics from other repetitive sequences since they are direct tandem repeats (Ei *et al*., 2016). Therefore, the mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR) molecular typing assay measures small open reading frames against 24 variable tandem repeat loci with specific primers complementary to the surrounding regions (Ei *et al*., 2016; Jeanes and O’Grady, 2016). This technique is mostly used together with spoligotyping, for contact and source tracing in patients with active TB (Jeanes and O’Grady, 2016).

Sequencing: Sequencing is an automated technique based on the principle of electrophoresis which identifies labelled DNA fragments (Nurwidya *et al*., 2018). The original chain termination method of sequencing described by Sanger has been overtaken by next generation sequencing (NGS) technologies which are slightly more complex (Jeanes and O'Grady, 2016). These NGS technologies range from targeted sequencing, deep sequencing, whole gene sequencing, to whole genome sequencing by a host of sequencing platforms (Jeanes and O'Grady, 2016; Iketleng *et al*., 2018; Colman *et al*., 2019). Sequencing is used to identify resistance profiles and novel mutations in *M. tuberculosis*. It has also been used to answer questions about TB transmission and has already become the routine method for *M. tuberculosis* typing in resource rich countries due to its superior resolution compared to conventional typing techniques (Iketleng *et al*., 2018; Colman *et al*., 2019). Various studies have shown that the relatedness of *M. tuberculosis* genomes can be estimated by comparing the single nucleotide polymorphism (SNP) differences between *M. tuberculosis* isolates using this technique (Jeanes and O'Grady, 2016; Iketleng *et al*., 2018; Colman *et al*., 2019).

Other techniques: There are other techniques that have not been discussed here such as; repetitive sequence based PCR (rep-PCR), amplified fragment length polymorphism (AFLP), polymorphic GC-rich repetitive RFLP (this alongside AFLP use the same principle as RFLP), and multi-locus sequence typing (MLST), since they are rarely used in TB diagnosis. With most of the techniques discussed above, *M. tuberculosis* DNA has proven to be a reliable biological signature for the identification and even the monitoring of TB disease.

2.2.3 Other biomarkers used in TB diagnosis

Generally, a biomarker refers to any molecule found in body fluids or tissues, which serves as a measurable indicator of either normal biological processes, the absence, presence, progress of disease, or the effects of treatment (Walzl *et al.*, 2011; Swanepoel *et al.*, 2015). Therefore, the reliability of a marker should depend on the consistency of its production, which is why most biomarkers in TB are host-specific and not pathogen-specific (Ota *et al*., 2014). The biomarkers currently being investigated in TB are centred on the measurement of the degree of TB infection (diagnosis), treatment efficacy (monitoring) and protective immune responses through vaccination (prevention) (Wallis *et al.*, 2013).

Despite several reports on new biomarker candidates, not many biomarkers have been independently validated for use and even fewer have been translated into new diagnostic tests (Gardiner *et al*., 2015; Goletti *et al*., 2016; Yerlikaya *et al*., 2017). This is not for lack of trying; attempts have been made to either provide insight into the TB disease condition (active TB versus latent TB infection) by using gene expression profiles as biomarkers, or to predict TB treatment response by using total white blood and macrophage cell counts. Unfortunately, many of these diagnostics are not validated, either because there are few or no follow-up studies, or due to the limited coordination and sharing of intellectual property between researchers (Brahmbhatt *et al*., 2006; Maertzdorf *et al*., 2011; Yerlikaya *et al*., 2017). A few more biomarkers and some of the techniques used in their assessment are cited below.

Volatile organic compounds: Volatile organic compounds (VOCs) represent a diverse group of carbon-based molecules that demonstrate distinct metabolic profiles characteristic of different microorganisms (Hong-Geller and Adikari, 2018). Upon infection, the VOCs released both by the pathogen and the infected host, could potentially be used as a diagnostic signature in determining the state of infection (Hong-Geller and Adikari, 2018).VOCs derived either from metabolites of *M. tuberculosis* or from products of oxidative stress in the host, have been isolated from breath as potential biomarkers of active pulmonary TB (Phillips *et al*., 2010; Swanepoel *et al.*, 2015).

Vascular endothelial growth factor: Vascular endothelial growth factor (VEGF) is a family of proteins that enhance granuloma formation via angiogenesis and vascularization (Kumar *et al*., 2016). Kumar and colleagues have previously shown that different sub-groups of VEGF (VEGF-A, VEGF-C and VEGF-R2) are important biomarkers of active TB disease severity and bacterial burden (Kumar *et al*., 2016). VEGF concentrations in patient plasma have also been shown to correlate positively with time to sputum conversion at 2 weeks of therapy (Riou *et al*., 2012). In addition, down-regulation of VEGF in sputum by anti-TB treatment, suggests that VEGF could serve as a biomarker in monitoring TB treatment response (Sigal *et al*., 2017).

T-cells: The expression of immune activation markers, CD38 and human leukocyte antigen-DR isotype (HLA-DR), on *M. tuberculosis*-specific CD4+ T-cells has been associated not only with *M. tuberculosis* load, but also with accurately distinguishing active from latent TB infection in blood-based studies (Adekambi *et al*., 2012). These signatures correctly classified individuals who had successfully completed chemotherapy, by correlating with a decrease in mycobacterial

load following treatment (Adekambi *et al*., 2012). T helper (Th)17 cells are also considered good potential biomarkers since they have a lengthy half-life, can become memory cells and have self-renewal capacities (Muranski *et al*., 2011; Kryczek *et al*., 2011; Goletti *et al.*, 2016).

Heat shock proteins: A heat shock protein (Hsp) is produced either by the host or *M. tuberculosis* in response to stress. They have been shown to carry out various tasks such as controlling protein degradation, heat tolerance, and immunomodulation, among others (Shekhawat *et al*., 2014). In *M. tuberculosis* infection, the Hsp are thought to elicit immune responses such as the activation of toll-like receptors (TLRs) (Shekhawat *et al*., 2014). Numerous Hsp in both host (Hsp 25, Hsp 60, Hsp 70, Hsp 90) and *M. tuberculosis* (Hsp 16, Hsp 65, Hsp 71) have been evaluated in TB patient sera, sputa and CSF (Shekhawat *et al*., 2014). These proteins were expressed as early as 48 hours after infection (Shekhawat *et al*., 2014). A derivative of Hsp 16, Hsp16.3, may also represent a potential biomarker of latent TB infection and novel pharmacological target for anti-TB drugs (Zhang *et al.*, 2018).

IP-10: The IFN-γ inducible protein 10 (IP-10) has been suggested for use as an alternative to IFN-γ, in the QuantiFERON TB Gold In-tube assay for the diagnosis of latent TB infection, since it is thought to be a more sensitive marker (Goletti *et al*., 2016). IP-10 levels were found to be higher in the plasma of children and adults with active TB, and in the urine of adult patients with TB (Goletti *et al*., 2016). It has been suggested as a confirmatory tool for clinical diagnosis and to guide specific therapy (den Hertog *et al*., 2015).

IgG antibodies: Antibodies have shown a lot of promise as potential biomarkers in the diagnosis of TB and in the monitoring of TB treatment response in patients. Proteomic studies have been used to identify antibodies against *M. tuberculosis* in patient sera and these antibody responses correlate with bacterial load (Walzl *et al*., 2011). High-affinity IgG crystallizable fragment (Fc) receptor IB, found to be the most differentially expressed gene in a transcriptomics study, could discriminate between active disease and latent TB infection (Maertzdorf *et al*., 2011). *M. tuberculosis*-specific IgG monoclonal antibodies have been isolated from the peripheral blood of healthy TB-exposed health care workers and are therefore potential biomarkers of immunity or resistance to infection (Zimmermann *et al*., 2016). However, according to Yerlikaya *et al*., (2017), little progress has been made in the use of antibodies since validation data are not being published, thereby widening the knowledge gap on the role antibodies play in the management of TB. Bridging the gap between the identification and validation of potential biomarkers is

essential in the development of simple diagnostics for use in resource-limited settings (Goletti *et al*., 2016).

Table 2.1 summarizes the various specimen types, appropriate assays and associated biomarkers/outcomes currently being explored for TB diagnosis.

Table 2.1: Diagnostic technique by specimen/organ type and test outcome

Diagnostic assay	Specimen type	Biomarker or test outcome
Tuberculin skin test (TST)	-	Delayed-type hypersensitivity to PPD
Chest X-ray	-	Lung lesions
Microscopy	Sputum, CSF	Tubercle bacilli
Solid culture (LJ)	Sputum	Tubercle bacilli
Liquid culture (MGIT 7H9 broth)	Sputum	Tubercle bacilli
LAM test	Urine	Lipoarabinomannan
PCR	Sputum, blood	IS6110 (TB RNA & DNA)
GeneXpert	Sputum, stool	IS6110, IS1081, *rpo*B RRDR
LPA	Sputum	TB RNA & DNA, *rpo*B, *kat*G, *inh*A
IGRA	Blood, CSF, urine	IFN-γ (ESAT-6, CFP-10), IP10
Chromatography	Sputum, plasma, breath	Mycolic acids, VEGF, VOCs
Mass spectrometry	Serum, urine	Hsp, MBL2, ITIH4-35K, miRNAs
Gel electrophoresis	Urine	TB RNA & DNA, proteins
LFA	Blood, saliva	MPT64 Ag, IgG antibodies
Flow cytometry	Blood, urine, BAL	CD4, CD27, CD38, Th17, TNF-α
RFLP	Sputum, blood	IS6110 (TB RNA & DNA)
Spoligotyping	Sputum, blood, urine	Spacer sequences in DR region
MIRU-VNTR	Sputum, blood, urine	ORFs in 24 tandem repeat loci
Sequencing	Sputum, blood	TB RNA & DNA, mutations, SNPs

LJ (Löwenstein-Jensen); LAM (lipoarabinomannan); PCR (polymerase chain reaction); LPA (line probe assay); IGRA (interferon gamma release assay); LFA (lateral flow assay); RFLP (restriction fragment length polymorphism); MIRU-VNTR (mycobacterial interspersed repetitive unit-variable number of tandem repeats); CSF (cerebrospinal fluid); BAL (broncho-alveolar lavage); PPD (purified protein derivative); IS6110 (insertion sequence 6110); TB (tuberculosis); RNA (ribonucleic acid); DNA (deoxyribonucleic acid); RRDR (rifampicin resistance-determining region); IFN-γ (interferon gamma); ESAT-6 (early secretory antigenic target-6); CFP-10 (culture filtrate protein-10); IP10 (inducible protein 10); VEGF (vascular endothelial growth factor); VOCs (volatile organic compounds); Hsp (heat shock protein); MBL2 (mannose-binding lectin 2); ITIH4-35K (inter-alpha-trypsin inhibitor heavy chain 4 precursor); miRNA (micro ribonucleic acid); MPT64 Ag (*Mycobacterium tuberculosis* complex antigenic protein 64); IgG (immunoglobulin gamma); CD4 (cluster of differentiation 4); TH17 (T helper 17); TNF-α (tumour necrosis factor alpha); DR (direct repeat); ORFs (open reading frames); SNPs (single nucleotide polymorphisms).

2.3 Advances and challenges in the treatment and prevention of tuberculosis

The effective prevention and treatment of TB is highly relevant in the control of its global burden, since it is responsible for disease in at least a third of the world's population (WHO, 2018). As is the case with TB diagnosis, several avenues have been attempted for the development of new vaccines as well as newer drugs for TB prevention and treatment respectively. However, this area has been slow in its progression compared to the area of TB diagnostics, due, in part, to the complexity of *M. tuberculosis* as a pathogen, as well as its interaction with the micro-environment in the host. Finding interventions that can target *M. tuberculosis* specifically without any detriment to the host within these complex niches has proven to be challenging.

2.3.1 New and re-purposed TB medications and their use in new short-course therapies

First-line anti-TB treatment is based on classical isoniazid, rifampicin, pyrazinamide and ethambutol therapy for drug susceptible *M. tuberculosis*. These drugs fall under Group 1 according to the old WHO classification, while group 2 - 5 drugs are used for drug resistant forms of *M. tuberculosis* (Tiberi *et al*., 2017). The newer drugs, linezolid, bedaquiline and delamanid have been indicated specifically for drug resistant *M. tuberculosis* (Tiberi *et al*., 2017). Bedaquiline, like rifampicin, targets both active and dormant *M. tuberculosis* while linezolid and delamanid, like pyrazinamide, demonstrate bactericidal and sterilising action against *M. tuberculosis* (Diacon *et al*., 2013; Caminero and Scardigli, 2015; Tiberi *et al*., 2017).

However, little is known about the effect of these antimicrobials on *M. tuberculosis* intra-macrophagic behaviour, and their impact on host cells. Studies by Genestet and colleagues claimed that isoniazid and bedaquiline impaired *M. tuberculosis* escape from the phagosome, rifampicin increased autolysosome formation, and linezolid and bedaquiline improved autophagy activation and efficacy (Genestet *et al.*, 2018). The data suggest that antimicrobials which favour autophagy activation may allow for better *M. tuberculosis* clearance by macrophages and could provide a basis for future anti-TB strategies (Genestet *et al.*, 2018).

Delamanid has been used in combination with bedaquiline for those with few treatment options such as the severely immunocompromised, those susceptible to so few drugs that a full

combination cannot be used, and those resistant to fluoroquinolones or injectables which constitute part of the regimen for the treatment of drug-resistant *M. tuberculosis* (Ferlazzo *et al*., 2018). However, concerns have arisen with regards to the side-effects (gastrointestinal, haematological, nervous system, hepatic) involved in using these new drugs although, one might argue that these side-effects can be controlled and are not life-threatening (Ferlazzo *et al*., 2018). Nevertheless, clinical trials are still underway to assess the safety and efficacy of some of these drug combinations: the US National Institutes of Health's AIDS Clinical Trials Group protocol (ACTG5343; NCT02583048) and the end-TB trial (NCT02754765) (Ferlazzo *et al*., 2018).

Drugs commonly used for diseases like diabetes, asthma, cancer, and arthritis, are now being repurposed for TB treatment (Zumla *et al*., 2016). Ibuprofen is one such drug and its use, even without standard anti-TB drugs, has been shown to reduce *M. tuberculosis* load and diminish inflammatory lung pathology in mice with active TB (Vilaplana *et al*., 2013; Kolloli and Subbian, 2017). Carbamazepine, an anticonvulsant, has been shown to enhance killing of *M. tuberculosis* in macrophages and reduce the multi drug resistant TB (MDR-TB) load in the lungs and spleens of mice (Schiebler *et al*., 2015). Similarly, drugs such as cilostazol, sildenafil and doxycycline have shown *M. tuberculosis* clearance in animal models (Kolloli and Subbian, 2017).

New regimens have been explored in recent years since many TB patients do not comply with treatment schedules due to their lengthy nature. Initially, chemo-sensitive TB was treated for 9 months, this was later shortened to 6 months and now, there are even considerations for 4 months of therapy (Sotgiu *et al*., 2015). Unfortunately, shortening treatment duration involves, in most cases, increasing dosing or adding more toxic drugs to the regimens leading to more severe side-effects. In most cases, rigorous research is not done and the decision to shorten treatment time is either based on the presence or absence of cavities on chest X-rays at the time of diagnosis or on an early treatment response (Johnson *et al*., 2009). These observations are clearly not enough as relapse rates have increased with 4 months of therapy from approximately 5 to 20% (Johnson *et al*., 2009; Colangeli *et al*., 2018). In order to minimize this risk, Colangeli and colleagues suggest minimum inhibitory concentration (MIC) measurements be carried out for patients, in order to inform the regimen to be assigned to them (4 months versus 6 months) (Colangeli *et al*., 2018).

2.3.2 Vaccines and host-directed therapies against TB

New scientific research strategies targeting the host and not the *M. tuberculosis* pathogen, are leading to the development of a wide range of therapies that modify human biological pathways so that they can in turn produce responses that will actively eliminate *M. tuberculosis* from the host (Zumla *et al.*, 2016). Several studies have shown that therapy involving host immune components which target processes such as autophagy, vitamin D pathways, and anti-inflammatory responses, can serve as adjunctive agents alongside the standard antimicrobials used in TB chemotherapy (Zumla *et al*., 2016; Kiran *et al.*, 2016; Kolloli and Subbian, 2017; Rekha *et al.*, 2018). Host-directed therapies (HDTs) are said to include safe and cheap drugs that are commonly used for non-infectious diseases, nutritional products, and cellular therapy (using the host's immune or mesenchymal stromal cells) (Zumla *et al.*, 2016). Such approaches are thought to be the best way forward for the eradication of TB, since these agents have the capacity to not only augment host cellular responses to *M. tuberculosis*, but also to target virulence factors, activate protective innate and adaptive immune responses, modulate excessive inflammation, and thereby reduce morbidity, mortality, and organ damage (Zumla *et al.*, 2016). The focus here is on a few potential HDTs which could be used in the near future.

Most studies aim to use HDTs as adjuncts to chemotherapy, and not replacement therapies (Kolloli and Subbian, 2017). The different kinds of HDTs used are cytokine therapy, antibody therapy, T-cell therapy, granuloma targeting, and autophagy induction therapy (Kolloli and Subbian, 2017). The data obtained from recent publications show that this approach has the potential to improve patient clinical outcomes and help reduce treatment duration (Kolloli and Subbian, 2017). Many vaccine studies have therefore been attempted for these reasons. An example is the use of *M. indicus pranii* (MIP) as a prophylactic or therapeutic vaccine in mice and guinea pigs challenged with *M. tuberculosis,* since both species share cross-reactive antigens (Singh *et al*., 2017). The administration of MIP led to significant reduction in *M. tuberculosis* bacillary loads and enhanced survival in these animals. Furthermore, a multi-centre clinical trial in TB patients with advanced disease, further confirmed MIP's immunotherapeutic role in these patients (Singh *et al*., 2017).

Bacille Calmètte-Guérin (BCG): BCG is currently being used both as a preventive and a therapeutic vaccine against TB. Preventive BCG is used in children against pulmonary TB, while therapeutic BCG has been explored as an adjunct to antimicrobial agents in order to shorten the

length of treatment and reduce the risk of relapse (Schon *et al*., 2013; Prabowo *et al*., 2019). Controversy surrounds BCG as an activator of humoral immunity since cell-mediated immunity (CMI) is thought to be solely responsible for protection against TB. Based on this assumption, a good TB vaccine should be one that induces a protective CD4+ T-cell response; the modified vaccinia virus Ankara expressing antigen 85A (MVA85A), one such vaccine, could unfortunately not confer the kind of protection provided by BCG, despite inducing antigen-specific T-cells (Tameris *et al*., 2013; Ndiaye *et al*., 2015). In a South African study, ELISPOT antigen-specific CD4+ T-cells did not appear to localize to the site of infection in lung tissue until 18 - 20 days after the establishment of disease (Jeyanathan *et al.*, 2014; Jacobs *et al.*, 2016). This implies there is a delay in the onset of CMI and it can therefore not solely be relied on for protection against *M. tuberculosis* infection.

Although there are issues surrounding the efficacy of BCG, it is still very much in use because there haven't been any major breakthroughs in the development of new vaccines against TB that are able to outperform BCG. For instance, the M72/AS01E (GlaxoSmithKline) candidate vaccine containing a mixture of two immunogenic *M. tuberculosis* antigens (Mtb32A and Mtb39A), a component of the malaria vaccine (RTS,S/AS01, GlaxoSmithKline) and a recombinant zoster vaccine (Shingrix, GlaxoSmithKline), showed a clinically acceptable safety profile and induced CMI in adults infected with *M. tuberculosis*, but could only provide 54.0% protection against active pulmonary TB (Van der Meeren *et al*., 2018). As drug resistance continues to spread, research aimed at improving BCG or new candidate vaccines is based on exploring combinations of TB vaccines with antimicrobial treatment, aiming for greater therapeutic value. Nevertheless, administering BCG as a therapeutic agent is a bit of a challenge due to the development of an exacerbated immune response (known as the "Koch phenomenon") in patients with active TB, especially in those with a high bacterial load. (Prabowo *et al*., 2019). Host-pathogen interactions should be considered when administering live organisms as vaccines. Hence, therapeutic BCG is advised to be administered following chemotherapy only after the bacterial load is significantly reduced, in order to allow for a synergistic effect (Prabowo *et al*., 2019). A study by Prabowo and colleagues used this kind of approach, by investigating immune responses in BCG-vaccinated human volunteers to whom antimicrobials (isoniazid and rifampicin) were administered. BCG vaccination was shown to significantly enhance the ability of isoniazid to control mycobacterial growth *ex vivo* (Prabowo *et al.*, 2019). Nevertheless, with BCG presenting

so many concerns due to its bacterial nature, safer avenues involving stimulating or boosting the host response are being explored.

MicroRNAs (miRNAs): These are small RNAs with non-coding properties that are involved in regulating various genes which are expressed in inflammatory and immune pathways (Liu *et al*., 2018; Sabir *et al*., 2018). MicroRNAs are potential diagnostic biomarkers in TB, since their expression during *M. tuberculosis* infection is said to provide information about the progression of disease and distinguishes active from latent TB infection (Wang *et al*., 2018; Sabir *et al*., 2018). Different microRNAs show different characteristics with regards to pathways like autophagy, which are involved in the elimination of *M. tuberculosis*; some miRNAs are promoters of these processes while others are inhibitors (Wang *et al*., 2018; Liu *et al*., 2018). However, Sabir *et al*., (2018) claim that the expression of these miRNAs can be manipulated for therapeutic purposes either through positive or negative regulation. MicroRNAs such as miR-155, miR-146a, miR-21, and miR-9, have been implicated in the activation of cells involved in the innate immune response (Sabir *et al*., 2018). MiR-125b inhibits TNF biosynthesis in human alveolar macrophages during *M. tuberculosis* infection while miR-146a regulates inflammatory responses in RAW264.7 macrophages and is a promoter of TLR signalling in murine cells (Sabir *et al*., 2018). Conversely, this miRNA is also involved in IFN-γ production in mouse models. The HDTs currently being employed are therefore meant to either restore miRNA expression or block the production of undesirable miRNAs, based on the kind of intervention required (Sabir *et al*., 2018).

Vitamin D3: Vitamin D3 (vitD3) is a dietary supplement with various immunomodulatory properties including the induction of antimicrobial peptides in lung epithelial cell lines, macrophages, and other human immune cells (Rekha *et al*., 2018). Vitamin D3 is responsible for inducing the production of cathelicidin and increasing the response to IFN-γ signalling, which enhances innate immune responses such as antigen processing and presentation (Zumla *et al*., 2016), the upregulation and expression of Atg5 and Beclin-1, which are inducers of autophagy (Kolloli and Subbian, 2017), and the suppression of pro-inflammatory cytokines and chemokines, thereby, accelerating the resolution of inflammatory responses during treatment (Kolloli and Subbian, 2017). It also plays a role in the suppression of cytotoxic T-cell proliferation and the enhancement of regulatory T-cell differentiation, thereby limiting tissue injury (Zumla *et al*., 2016; Kolloli and Subbian, 2017). It has also been implied to inhibit lipid

accumulation in *M. tuberculosis*-infected macrophages, thereby reducing fatty acid synthesis in the organism which eventually leads to its starvation (Kolloli and Subbian, 2017). Vitamin D3 is generally used in conjunction with agents such as phenylbutyrate (Zumla *et al*., 2016; Rekha *et al*., 2018). Rekha and colleagues showed that the use of phenylbutyrate and vitD3 as adjunct therapy to standard anti-TB treatment improved treatment outcomes in TB patients. Vitamin D3 also demonstrated favourable immunomodulatory and autophagic properties, since there was a significant decline in the concentration of some of pro-inflammatory cytokines, chemokines and mitogens as well as an increase in the expression of autophagy markers when vitD3 was used alone (Rekha *et al*., 2018).

Antibodies and their role in TB treatment: Antibodies are derived from immunoglobulins, which are proteins with a large number of binding sites that accommodate a variety of molecules, including antigen and complement receptor binding (Schroeder Jr and Cavacini, 2010). This binding creates complexes that when activated, can lead to the elimination of bacteria Initially, antibodies were only used as biomarkers for TB diagnosis and not as a therapeutic strategy, since antibodies have not traditionally been thought to be an important component of the immune response against intracellular pathogens such as *M. tuberculosis* (Jacobs *et al.*, 2016). The conflicting knowledge on the protectiveness of the immune response elicited by antibodies and the evidence for protectiveness mostly shown in the literature to be passive, by the demonstration of disease in the absence of antibodies, rather than the demonstration of active protection by the presence of these antibodies, has not helped the case for antibodies as good therapeutics against TB (Ziegenbalg *et al*., 2013; Jacobs *et al.*, 2016). The current sanctioned TB BCG vaccine is still based on CMI, perhaps owing to the fact that humoral immune responses are thought to be an evasion mechanism used by TB to steer host responses away from a more protective Th1-mediated cytotoxic response (Ashenafi *et al*., 2014; Jacobs *et al*., 2016).

Monoclonal antibodies (IgG): On a positive note, some studies have revealed the direct contribution of antibodies (especially IgG monoclonal antibodies) to the decrease in severity of TB infection (Cobat *et al*., 2009; Encinales *et al*., 2010; Fletcher *et al.*, 2016). Some individuals in high-exposure settings show increased levels of IgG against *M. tuberculosis* even after extended periods of time in close contact with active TB patients. These individuals do not have any detectable T-cell response to *M. tuberculosis* antigens by either TST or *ex vivo* stimulation (Cobat *et al*., 2009; Encinales *et al*., 2010). In a study conducted by Fletcher *et al*., (2016), high

levels of IgG against Ag85A were associated with a reduced risk of disease in infants. Abebe and colleague claim that sera from immunized mice reduce bacillary load in mice with partially treated *M. tuberculosis* infection by 100-fold and pulmonary infiltration by 3-fold; evidence of monoclonal antibody protection is proven by the lack of T-cells in the sera of these mice (Abebe and Bjune, 2009). Furthermore, IgG monoclonal antibodies said to be specific for arabinomannan, have been shown to prolong the survival of mice infected with lethal doses of *M. tuberculosis*, by enhancing granuloma formation and nitric oxide production in the infected cells (Jacobs *et al.*, 2016).

Immunoglobulin gamma (IgG) is the most abundant monoclonal antibody in plasma, since it is produced by plasma cells derived from B-cells that have undergone class switching (Schroeder and Cavacini, 2010). It is also found in mucosal secretions (though to a lesser extent as compared to immunoglobulin alpha-IgA), predominantly in the lower respiratory tract (Plotkin, 2008; Abebe and Bjune, 2009).

Figure 2.1 (*downloaded from www.researchgate.net on 15th February 2020 [Rodrigo et al., 2015] - CC BY 4.0*) depicts a mature immunoglobulin gamma monoclonal antibody, alongside its various domains. IgG consists of two light chains and two heavy chains (gamma), and each light chain-heavy chain pair is held together by chemical bonds, forming the variable fragment (Fv) of the antibody that is responsible for antigen-recognition and -binding (Rodrigo *et al*., 2015; Herold *et al*., 2017). Based on the number of variable domains, IgG has two antigen-binding sites available (bivalent). The single-chain variable fragment (scFv) is the entire antigen-binding site of the antibody and is responsible for the specificity of the antibody, due to its affinity for a single antigen (monovalent) (Rodrigo *et al*., 2015; Chiu *et al*., 2019). The crystallizable fragment (Fc) is made up of constant domains which help stabilize the antibody, increase serum antibody half-life, and enhance antibody interaction with other components of the adaptive immune system, for effects such as complement fixation and immune cell activation (Herold *et al*., 2017; Chiu *et al*., 2019).

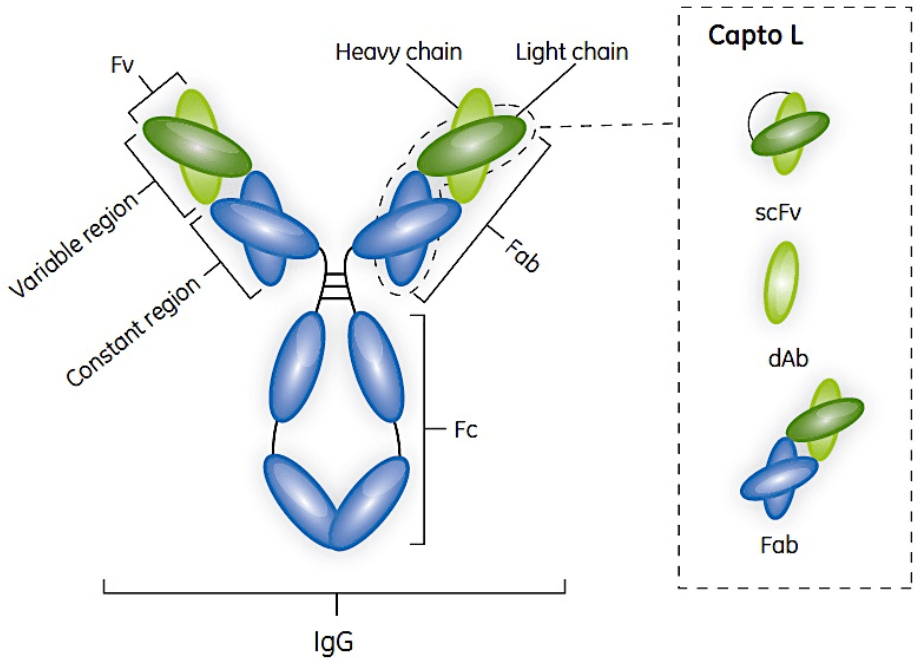

Figure 2.1: The structure of an immunoglobulin gamma (IgG) monoclonal antibody

Fv (variable fragment); Fab (antigen-binding fragment); Fc (constant fragment); Capto L (a matrix containing the immunoglobulin binding protein L ligand, that binds to the variable region of the kappa light chain without interfering with the antigen binding site of the monoclonal antibody); scFv (single chain variable fragment); dAb (domain antibody).

In the course of infection, antibody development seems to be influenced by several factors. These include: the stage of infection, co-infection, and host genotype which is probably why antibody protection varies from one individual to another (Jacobs *et al.*, 2016). When it comes to the effectiveness of monoclonal antibodies, factors such as sub-class (isotype), epitope binding (antigenic target binding), and ability to opsonize *M. tuberculosis*, are important and ought to be considered together (Jacobs *et al.*, 2016). With regards to the first factor, a study where two immunoglobulin mu (IgM) monoclonal antibodies could not enhance clearance of *M. tuberculosis* in mice despite their validated surface binding, confirmed the importance of antibody isotype in conferring protection (Jacobs *et al*., 2016). The structural and functional differences in the constant regions of the heavy chain of each monoclonal antibody are responsible for its effectiveness (Abebe *et al*., 2018). Secondly, monoclonal antibodies can be assessed for their capacity to bind to mycobacterial epitopes (the part of the antigen specific to the monoclonal antibody) in order to determine their complementarity, as well as their protective potential both *in vitro* and *in vivo*. This assessment can also aid in the manipulation of target

binding sites on novel monoclonal antibodies to improve antigenic binding (Sei *et al*., 2019). Thirdly, producing a therapeutic vaccine containing antibodies capable of opsonizing *M. tuberculosis* is a potential approach to obtaining antibody-mediated immunity in TB (Perley *et al*., 2014; Jacobs *et al*., 2016). The effect of antibody opsonization in the phagocytosis of *M. tuberculosis* has been investigated in the past (Armstrong and Hart, 1975). Armstrong and Hart used immunized rabbit serum to compare phagocytosis of both opsonized and non-opsonized bacteria and concluded that immunized serum enhanced phagolysosome fusion in *M. tuberculosis* infection (Armstrong and Hart, 1975). In this light, IgG has been implicated in the precipitation, agglutination, and neutralization of bacteria (Ballow, 2011). Its ability to opsonize said bacteria in preparation for the process of phagocytosis, together with its high level of specificity, make this monoclonal antibody desirable (Ballow, 2011; Senoputra *et al*. 2015). The IgG isotype which is the most predominant in the human body and has the longest serum half-life, consists of the four sub-classes IgG1-IgG4 (Schroeder Jr and Cavacini, 2010). Among these sub-classes, IgG1 is the most effective overall, since it can fix complement component c1q used for phagocytosis by granulocytes (IgG4 cannot), and has affinity for all three FcR classes (IgG2 and IgG4 do not bind to all three classes, and IgG3 has a weaker affinity) (Schroeder Jr and Cavacini, 2010).

Nanoparticles: A new approach currently being explored in vaccine therapy is nanoparticle delivery via inhalation. The idea is to activate *M. tuberculosis*-containing macrophages in the lungs, by HDT using Magainin-I analog peptide (MIAP) nano-formulations, in order to study important regulators of antimicrobial responses in host cells (Sharma *et al*., 2018). A few studies have shown that this approach is feasible (Dacoba *et al*., 2017; Sabir *et al*., 2018; Sharma *et al*., 2018). Typically, the size range for inhalable particles for local lung delivery is between 1 and 5 microns, whereas nano-size particles (which are less than 1 micron) are easily exhaled during the breathing process (Sharma *et al*., 2018). Therefore, precise particle engineering is crucial for the controlled release and the maximum deposition of drugs for an optimal therapeutic outcome when inhaled (Sabir *et al*., 2018; Sharma *et al*., 2018). Nano-particle-mediated delivery of substances or compounds of interest is another valid foundation for future HDTs in TB. Techniques capable of encapsulating substances (such as miRNAs and monoclonal antibodies) and delivering them to cells such as macrophages (the first line of defence) that have the innate ability to internalize foreign bodies, are currently being investigated (Sabir *et al*., 2018). This

type of technology has been used in a proof of principle *M. tuberculosis* vaccination strategy combining an anti-LAM antibody with a baculoviral delivery system in mice (Shin *et al*., 2017).

2.3.3 Processes involved in host-directed therapies against TB: Autophagy

Autophagy is a broad concept that involves a number of metabolic, physiological, immunological (mostly involving cytokines and innate immune signalling), and pharmacological (antimicrobial) processes which present contents of the host cytoplasm to lysosomes for degradation (Mizushima *et al*., 2011). Autophagy, simplified, begins with the formation of double-membrane structures called phagophores that surround the cytoplasmic contents of interest and expand to form autophagosomes (Sabir *et al.*, 2018). The autophagosomes fuse with lysosomes after the production of several biochemical agents, to produce an organelle called an autolysosome, which is responsible for degrading the contents of interest (Deretic, 2014; Siqueira *et al*., 2018).

Two forms of autophagy have been identified; nonselective (bulk or generalized) autophagy which involves the degradation of the cytoplasm, and selective autophagy, which involves the capture of specific cytosol targets by autophagosomes, upon their recognition by autophagic receptors (Birgisdottir *et al*., 2013; Deretic, 2014). The autophagic process is thought to be triggered by the host to rid itself of TB by activating macrophages via exogenous stimulations, such as rapamycin, vitD3, and sometimes even IFN-γ (Gutierrez *et al*., 2004; Rekha *et al*., 2012; Seto *et al*., 2013; Siqueira *et al*., 2018). The stimulation of autophagy has the potential to enhance the immune response of the host and offers new strategies for developing immunogens that may improve protection upon immunization (Flores-Valdez *et al.*, 2018). *M. tuberculosis* is mostly an intracellular pathogen, and autophagy is an essential component of the immune response towards its elimination; it has been shown to act as a mechanism that contributes to *M. tuberculosis* clearance both *in vitro* and *in vivo* (Seto *et al*., 2013; Siqueira *et al*., 2018; Genestet *et al.*, 2018).

As an important component of the host innate and adaptive immune responses, macrophage autophagy plays a crucial role in host protection against TB (Zhang *et al.*, 2018). However, dormant bacilli can escape from the autophagosome, thereby surviving within the host for an extended period, although the underlying mechanism(s) is unknown (Zhang *et al.*, 2018).

Mechanisms employed by M. tuberculosis to evade the autophagic process

Autophagy is a complex process which involves several pathways, numerous biochemical agents, and markers such as coronin-1a, cardiolipin, reactive oxygen species, TLRs, and IFN genes (Singh *et al*., 2010; Li *et al*., 2011; Seto *et al*., 2013). *M. tuberculosis* has devised a few strategies to evade autophagy, for instance, Hsp16.3, one of the main proteins expressed during latent TB infection, is employed by the organism since it helps maintain protein stability and its long-term survival (Zhang *et al.*, 2018). *M. tuberculosis* also manoeuvres the intricate process of autophagy by using the expression of miRNAs in order to create a favourable replicative environment (Sabir *et al.*, 2018). The interaction between *M. tuberculosis* and host macrophages induces the expression of miR-33 and miR-33* which reduces mitochondrial fatty acid oxidation and lipophagy (the autophagy of lipid droplets), hence increasing cellular lipid content, essential for *M. tuberculosis* as a nutrient source (Ouimet *et al*., 2016; Sabir *et al.*, 2018; Siqueira *et al*., 2018). Similarly, the increase in miR-30A expression during infection with *M. tuberculosis* decreases beclin-1 expression levels, resulting in the inhibition of autophagosome production in order to promote the intracellular survival of *M. tuberculosis* (Chen *et al*., 2015; Sabir *et al.*, 2018). Furthermore, miR-144* inhibits the work of antimicrobial agents against *M. tuberculosis* in macrophages by targeting the expression of DNA damage-regulated autophagy modulator 2, thereby enhancing *M. tuberculosis* replication (Kim *et al*., 2017; Sabir *et al.*, 2018).

2.3.4 Processes involved in host-directed therapies against TB: Phagocytosis

Phagocytosis is a process which involves the engulfment and degradation of bacterial components. It has been described as the recognition of pathogens greater than 0.5 micron in size by phagocytic cells through specific pathogen-recognition receptors (PRRs), the formation of a phagosome by these phagocytic cells, and the fusion of the newly formed phagosome to a lysosome in order to degrade the bacteria with lysosomal contents such as lysozymes and other oxidants (Richards and Endres, 2014; Rodriguez *et al*., 2017). **Figure 2.2** *(copied from https://iqbiosciences.com on 13th March 2019 [Jean Lee, graphic artist, IQ Biosciences])* is a diagrammatic representation of the phagocytic process.

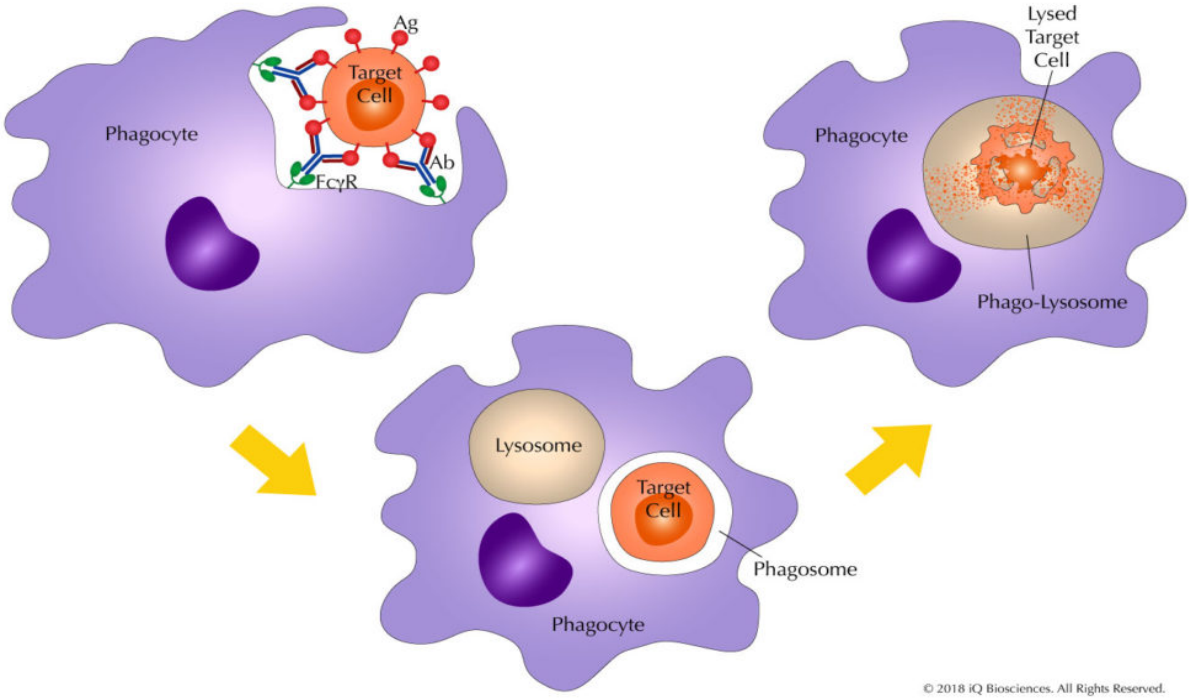

Figure 2.2: The phagocytic process

There is some debate as to which cells are the most effective phagocytes of *M. tuberculosis*. Some schools of thought claim that dendritic cells (DCs) are more effective, while others are of the opinion that neutrophils are the most effective cells (Seto *et al.*, 2013; Bangani *et al.*, 2016). Seto *et al.*, (2013) claim that mycobacterial autophagosome markers mobilize around DCs and not macrophages, for the initiation of phagolysosome fusion. They further hypothesize that this process helps DCs to be better antigen-presenters to major histocompatibility complex (MHC) class II for mycobacterial elimination. Bangani *et al.*, (2016) on the other hand, claim that neutrophils are better phagocytes of *M. tuberculosis,* although, Alvarez-Jiménez and colleagues argue that even though neutrophils are the most abundant phagocytic cells in humans, they only secrete extracellular vesicles which help present TB to macrophages, thereby enhancing macrophage phagocytosis (Alvarez-Jiménez *et al.*, 2018).

Antibodies and the phagocytic process

Antibodies can protect against various intracellular pathogens by modulating immunity through Fc receptor-mediated phagocytosis (FcR-P) (Jacobs *et al.*, 2016). The antibodies that contribute

to host immunity against *M. tuberculosis* are most likely those that opsonize the bacillus and modulate the host macrophage response to FcR-P (Kumar *et al.*, 2015; Jacobs *et al.*, 2016). Therefore, the process of opsonization by antibodies for FcR-mediated phagocytosis appears to be a major mechanism of protection in models of *M. tuberculosis* infection (Jacobs *et al.*, 2016). FcR-P, though common, is not the only mechanism of antibody-mediated immunity that has been explored in TB. Antibody opsonization also enhances complement-induced killing via the activation of host membrane attack complexes (Jacobs *et al.*, 2016). However, simply using antibodies and complement independently or in combination is not good enough. Cai and colleagues claim that activated complement component C1q could rather be enhancing the uptake of bacilli (Cai *et al.*, 2014). It therefore seems highly unlikely that the human immune response produces potently neutralizing antibodies capable of killing or inhibiting the growth of *M. tuberculosis* independently of phagocytes (Cai *et al.*, 2014; Jacobs *et al.*, 2016). **Figure 2.3** (*downloaded from www.researchgate.net on 13th July 2020 [Jacobs et al., 2016] - CC BY 4.0*) demonstrates FcR-P of *M. tuberculosis*.

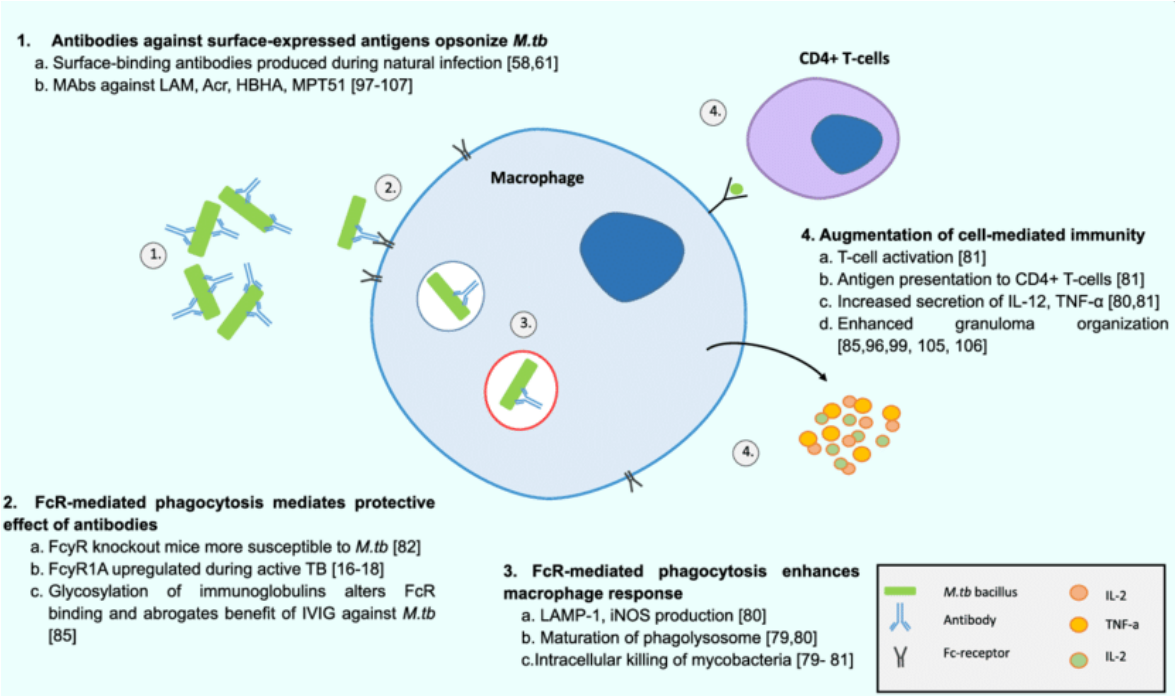

Figure 2.3: FcR-mediated phagocytosis
(references quoted in the Figure are applicable to the original publication)

A 2005 study demonstrated the effectiveness of antibodies as good opsonins for phagocytosis and enhancers of CMI by showing that anti-LAM antibodies could be used to pre-treat *M. tuberculosis* bacilli, enhance their uptake by macrophages, and increase the production of CD4+

and CD8+ T-cells (De Valliere *et al*., 2005). This finding has led to more recent advancements; in a study conducted in India, sera from naturally-infected healthy donors showed elevated levels of opsonizing antibodies which were able to enhance the intracellular killing of *M. tuberculosis* strain H37Rv in macrophages, by increasing LAMP-1 and nitric oxide species localization to the phagosome, as well as phagolysosome acidification (Kumar *et al.*, 2015). Recent work has described another phagolysosomal pathway called light chain 3 (LC3)-associated phagocytosis (LAP), which links conventional phagocytosis and autophagy by recruiting LC3, an enhancer of phagosome-lysosome fusion (Cemma and Brumell, 2012; Deretic, 2014). LAP is initiated by macrophage PRRs and TLRs since LC3 associates with *M. tuberculosis*-containing phagosomes and requires NADPH oxidase (Lee *et al*., 2010; Martinez *et al*., 2011; Köster *et al.*, 2018).

Mechanisms employed by M. tuberculosis to evade the phagocytic process

Köster *et al.*, (2018) discovered that CpsA, an exported *M. tuberculosis* virulence factor, blocks LAP by interfering with the recruitment of cytochrome b-245 beta polypeptide (CYBB/NOX2) to the phagosome. In both macrophages and mice with *cps*A- deficient *M. tuberculosis*, the bacteria were successfully cleared by NADPH oxidase and LC3-associated lysosomal trafficking (Köster *et al.*, 2018). CpsA belongs to a family, known for its enzymatic role in cell wall assembly in gram-positive bacilli (Köster *et al.*, 2018). However, data suggest that CpsA inhibits CYBB oxidase independently of cell wall function (Köster *et al.*, 2018). Thus, CpsA may have evolved from an enzyme involved in cell wall integrity to a significant virulence factor that *M. tuberculosis* uses to evade the innate immune response (Köster *et al.*, 2018). More studies support these findings by demonstrating the modulation of Rab GTPase transport (responsible for phagosome maturation) by *M. tuberculosis*, thus resulting in the inhibition of phagolysosome biogenesis in macrophages (Sugaya *et al*., 2011; Seto *et al*., 2013).

2.4 Conclusion

Tuberculosis is a major global problem and the causative agent, *M. tuberculosis,* is a resilient pathogen. It is constantly evolving, exhibiting novel mechanisms of resistance in order to evade arrest and elimination, and to thrive in a self-protected, replicative micro-environment. In this overview, the focus was centred on new diagnostics, novel therapeutics and the pathways these therapies adopt towards eradicating TB or at least limiting its spread and reactivation in humans. With the current understanding of *M. tuberculosis* complexity, as discussed in the literature,

therapy that cuts across most, if not all forms and pathways of pathogen resistance, is desirable as an intervention against TB. In conclusion, HDTs might hold the key to better control of recurrent TB. In the ensuing chapters, the potential of a novel monoclonal antibody, JG7, is considered in this context.

2.5 References

Abebe F, Bjune G (2009) The protective role of antibody responses during *Mycobacterium tuberculosis* infection. *Clinical and Experimental Immunology* **157**: 235–43.

Abebe F, Belay M, Legesse M, KLMCF, Ottenhoff THM (2018) IgA and IgG against *Mycobacterium tuberculosis* Rv2031 discriminate between pulmonary tuberculosis patients, *Mycobacterium tuberculosis*-infected and noninfected individuals. *PLoS One* **13**(1): e0190989.

Adekambi T, Ibegbu CC, Kalokhe AS, Yu T, Ray SM, Rengarajan J (2012) Distinct effector memory CD4+ T cell signatures in latent *Mycobacterium tuberculosis* infection, BCG vaccination and clinically resolved tuberculosis. *PLoS One* **7**: e36046.

Alvarez-Jiménez DV, Leyva-Paredes K, García-Martínez M, Vázquez-Flores L, García-Paredes GV, Campillo-Navarro M, Romo-Cruz I, Rosales-García HV, Castañeda-Casimiro J, González-Pozos S, Hernández MJ, Wong-Baeza C, García-Pérez EB, Ortiz-Navarrete V, Estrada-Parra S, Serafín-López J, Wong-Baeza I, Chacón-Salinas R, Estrada-García I (2018) Extracellular vesicles released from *Mycobacterium tuberculosis*-infected neutrophils promote macrophage autophagy and decrease intracellular mycobacterial survival. *Frontiers in Immunology* **9**: 272.

Armstrong JA, Hart P (1975) Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual non-fusion pattern and observations on bacterial survival. *Experimental Medicine* **142**: 1–16.

Ashenafi S, Aderaye G, Bekele A, Zewdie M, Aseffa G, Hoang ATN, Carow B, Habtamu M, Wijkander M, Rottenberg M, Aseffa A, Andersson J, Svensson M, Brighenti S (2014) Progression of clinical tuberculosis is associated with a Th2 immune response signature in combination with elevated levels of SOCS3. *Clinical Immunology* **151**: 84–99.

Ayer M, Harm-Anton K (2017) Cell-mediated delivery of synthetic nano and microparticles. *Journal of Controlled Release* **259**: 92–104.

Ballow M (2011) The IgG molecule as a biological immune response modifier: Mechanisms of action of intravenous immune serum globulin in autoimmune and inflammatory disorders. *Journal of Allergy and Clinical Immunology* **127**(2): 315–323.

Bangani N, Nakiwala J, Martineau RA, Wilkinson JR, Wilkinson AK, Lowe MD (2016) HIV-1 Infection Impairs CD16 and CD35 Mediated Opsonophagocytosis of *Mycobacterium tuberculosis* by Human Neutrophils. *Journal of Acquired Immune Deficiency Syndrome* **73**: 263–267.

Birgisdottir AB, Lamark T, Johansen T (2013) The LIR motif-Crucial for selective autophagy. *Journal of Cell Science* **126**: 3237–3247.

Boehme CC, Nabeta P, Hillerman D, Nicol MP, Shenai S, Krapp F, Allen J, Tahirli R, Blakemore R, Rustomjee R, Milovic A, Jones M, O'Brien SM, Persing DH, Ruesch-Gerdes S, Gotuzzo E, Rodrigues C, Alland D, Perkins MD (2011) Rapid molecular detection of tuberculosis and rifampin resistance. *New England Journal of Medicine* **363**: 1005–1015.

Brahmbhatt S, Black GF, Carroll NM, Beyers N, Salker F, Kidd M, Lukey PT, Duncan K, van Helden P, Walzl G (2006) Immune markers measured before treatment predict outcome of intensive phase tuberculosis therapy. *Clinical and Experimental Immunology* **146**: 243–252.

Cai Y, Yang Q, Tang Y, Zhang M, Liu H, Zhang G, Deng Q, Huang J, Gao Z, Zhou B, Feng CG, Chen X (2014) Increased complement C1q level marks active disease in human tuberculosis. *PLoS One* **9**: e92340.

Caminero JA, Scardigli A (2015) Classification of antituberculosis drugs: a new proposal based on the most recent evidence. *European Respiratory Journal* **46**: 887–93.

Cemma M, Brumell JH (2012) Interactions of pathogenic bacteria with autophagy systems. *Current Biology* **22**: R540–R545.

Chen Z, Wang T, Liu Z, Zhang G, Wang J, Feng S, Liang J (2015) Inhibition of autophagy by MiR-30A induced by mycobacteria tuberculosis as a possible mechanism of immune escape in human macrophages. *Japanese Journal of Infectious Diseases* **68**: 420–4.

Chiu LM, Goulet RD, Teplyakov A, Gilliland LG (2019) Antibody Structure and Function: The Basis for Engineering Therapeutics. *Antibodies (Basel)* **8**(4): 55.

Cobat A, Gallant CJ, Simkin L, Black GF, Stanley K, Hughes J, Doherty TM, Hanekom WA, Eley B, Jaïs J-P (2009) Two loci control tuberculin skin test reactivity in an area hyperendemic for tuberculosis. *Experimental Medicine* **206**: 2583–9.

Colangeli R, Jedrey H, Kim S, Connell R, Ma S, Chippada Venkata DU, Chakravorty S, Gupta A, Sizemore EE, Diem L, Sherman RD, Okwera A, Dietze R, Boom HW, Johnson LJ, MacKenzie RW, Alland D for the DMID 01-009/Tuberculosis Trials Consortium Study 22 Teams (2018) Bacterial Factors That Predict Relapse after Tuberculosis Therapy. *New England Journal of Medicine* **379**: 823–33.

Colman ER, Mace A, Seifert M, Hetzel J, Mshaiel H, Suresh A, Lemmer D, Engelthaler MD, Catanzaro GD, Young GA, Denkinger MC, Rodwell CT (2019) Whole-genome and targeted sequencing of drug-resistant *Mycobacterium tuberculosis* on the iSeq100 and MiSeq: A performance, ease-of-use, and cost evaluation. *PLoS Medicine* **16**(6): e1002823.

Conte Junior AC, de la Torre LAC, Figueiredo de SEE, Lilenbaum W, Paschoalin FMV (2015) Application of High Performance Liquid Chromatography for Identification of Mycobacterium spp. *Web of Science™ Core Collection* http://dx.doi.org/10.5772/59630 accessed on 5th April, 2019.

Dacoba TG, Olivera A, Torres D, Crecente-Campo J, Alonso MJ (2017) Modulating the immune system through nanotechnology. *Seminars in Immunology* **34**: 78–102.

Davies LJ, Cattamanchi A, Cuevas EL, Hopewell PC, Steingart KR (2013) Diagnostic accuracy of same-day microscopy versus standard microscopy for pulmonary tuberculosis: a systematic review and meta-analysis. *Lancet Infectious Diseases* **13**: 147–54.

De Valliere S, Abate G, Blazevic A, Heuertz RM, Hoft DF (2005) Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. *Journal of Infection and Immunity* **73**: 6711–20.

Den Hertog AL, Montero-Martin M, Saunders RL, Blakiston M, Menting S, Sherchand BJ, Lawson L, Oladimeji O, Abdurrahman TS, Cuevas EL, Anthony MR (2015) Cytokine kinetics in the first week of tuberculosis therapy as a tool to confirm a clinical diagnosis and guide therapy. *PLoS One* **10**: e0129552.

Deretic V (2014) Autophagy in Tuberculosis. *Cold Spring Harbor Perspectives in Medicine* **4**: a018481.

Diacon AH, Dawson R, Von Groote-Bidlingmaier F, Symons G, Venter A, Donald PR, Conradie A, Erondu N, Ginsberg AM, Egizi E, Winter H, Becker P, Mendel CM (2013) Randomized dose-ranging study of the 14-day early bactericidal activity of bedaquiline (TMC207) in patients with sputum microscopy smear-positive pulmonary tuberculosis. *Antimicrobial Agents and Chemotherapy* **57**: 2199–203.

Doherty M, Wallis SR, Zumla A, WHO–Tropical Disease Research/European Commission joint expert consultation group (2009) Biomarkers for tuberculosis disease status and diagnosis. *Current Opinion in Pulmonary Medicine* **15**: 000–000.

Ei WP, Aung WW, Lee SJ, Choi G-E, Chang LC (2016) Molecular Strain Typing of *Mycobacterium tuberculosis*: A Review of Frequently Used Methods. *Journal of Korean Medical Sciences* **31**: 1673–1683.

Encinales L, Zuniga J, Granados-Montiel J, Yunis M, Granados J, Almeciga I, Clavijo O, Awad C, Collazos V, Vargas-Rojas MI (2010) Humoral immunity in tuberculin skin test anergy and its role in high-risk persons exposed to active tuberculosis. *Molecular Immunology* **47**: 1066–73.

Fanny M-L, Beyam N, Gody CJ, Zandanga G, Yango F, Manirakiza A, Rigouts L, Pierre-Audigier C, Gicquel B, Bobossi G (2012) Fine-needle aspiration for diagnosis of tuberculous lymphadenitis in children in Bangui, Central African Republic. *BMC Pediatrics* **12**: 191.

Ferlazzo G, Mohr E, Laxmeshwar C, Hewison C, Hughes J, Jonckheere S, Khachatryan N, De Avezedo V, Egazaryan L, Shroufi A, Kalon S, Cox H, Furin J, Isaakidis P (2018) Early safety and efficacy of the combination of bedaquiline and delamanid for the treatment of patients with drug-resistant tuberculosis in Armenia, India, and South Africa: a retrospective cohort study. *Lancet Infectious Diseases* **18**: 536–44.

Fletcher HA, Snowden MA, Landry B, Rida W, Satti I, Harris SA, Matsumiya M, Tanner R, O'Shea MK, Dheenadhayalan V, Bogardus L, Stockdale L, Marsay L, Chomka A, Harrington-Kandt R, Manjaly-Thomas ZR, Naranbhai V, Stylianou E, Darboe F, Penn-Nicholson A, Nemes E, Hatheril M, Hussey G, Mahomed H, Tameris M, McClain JB, Evans TG, Hanekom WA, Scriba TJ, McShane H (2016) T-cell activation is an immune correlate of risk in BCG vaccinated infants. *Nature Communications* **7**: 11290.

Flores-Valdez AM, Segura-Cerda AC, Gaona-Bernal J (2018) Modulation of autophagy as a strategy for development of new vaccine candidates against tuberculosis. *Molecular Immunology* **97**: 16–19.

Fuhrmann S, Streitz M, Kern F (2008) How Flow Cytometry is Changing the Study of TB Immunology and Clinical Diagnosis. *Cytometry Part A* **73A**: 1100–1106.

Gardiner JL, Karp CL (2015) Transformative tools for tackling tuberculosis. *Journal of Experimental Medicine* **212**(11): 1759–69.

Genestet C, Bernard-Barret F, Hodille E, Ginevra C, Ader F, Goutelle S, Lina G, Dumitrescu O on behalf of the Lyon TB study group (2018) Antituberculous drugs modulate bacterial phagolysosome avoidance and autophagy in *Mycobacterium tuberculosis*-infected macrophages. *Tuberculosis* **111**: 67–70.

Goletti D, Petruccioli E, Joosten AS, Ottenhoff HMT (2016) Tuberculosis biomarkers: from diagnosis to protection. *Infectious Disease Reports* **8**: 6568.

Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V (2004) Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* **119**: 753–66.

Hare NJ, Chan B, Chan E, Kaufman KL, Britton WJ, Saunders BM (2015) Microparticles released from *Mycobacterium tuberculosis*-infected human macrophages contain increased levels of the type I interferon inducible proteins including ISG15. *Proteomics* **15**(13).

Herold ME, John C, Weber B, Kremser S, Eras J, Berner C, Deubler S, Zacharias M, Buchner J (2017) Determinants of the assembly and function of antibody variable domains. *Scientific Reports* **7**: 12276.

Hong-Geller E, Adikari S (2018) Volatile Organic Compound and Metabolite Signatures as Pathogen Identifiers and Biomarkers of Infectious Disease. *IntechOpen* http://dx.doi.org/10.5772/intechopen.72398 accessed on 17th March 2019.

Iketleng T, Lessells R, Dlamini TM, Mogashoa T, Mupfumi L, Moyo S, Gaseitsiwe S, de Oliveira T (2018) *Mycobacterium tuberculosis* Next-Generation Whole Genome Sequencing: Opportunities and Challenges. *Tuberculosis Research and Treatment* doi: https://doi.org/10.1155/2018/1298542.

Jacobs JA, Mongkolsapaya J, Screaton RG, McShane H, Wilkinson JR (2016) Antibodies and tuberculosis. *Tuberculosis* **101**: 102–113.

Jeanes C, O'Grady J (2016) Diagnosing tuberculosis in the 21st century – Dawn of a genomics revolution? *International Journal of Mycobacteriology* **5**(4): 384–391.

Jeyanathan M, McCormick S, Lai R, Afkhami S, Shaler CR, Horvath CN, Damjanovic D, Zganiacz A, Barra N, Ashkar A, Jordana M, Aoki N, Xing Z (2014) Pulmonary *M. tuberculosis* infection delays Th1 immunity via immunoadaptor DAP12-regulated IRAK-M and IL-10 expression in antigen-presenting cells. *Mucosal Immunology* **7**: 670–83.

Johnson JL Hadad DJ, Dietze R, Maciel EL, Sewali B, Gitta P, Okwera A, Mugerwa RD, Alcaneses MR, Quelapio MI, Tupasi TE, Horter L, Debanne SM, Eisenach KD, Boom WH

(2009) Shortening treatment in adults with noncavitary tuberculosis and 2-month culture conversion. *American Journal of Respiratory and Critical Care Medicine* **180**: 558–563.

Kim JK, Lee HM, Park KS, Shin DM, Kim TS, Kim YS, Suh HW, Kim SY, Kim IS, Kim JM, Son JW, Sohn KM, Jung SS, Chung C, Han SB, Yang CS, Jo EK (2017) MIR144* inhibits antimicrobial responses against *Mycobacterium tuberculosis* in human monocytes and macrophages by targeting the autophagy protein DRAM2. *Autophagy* **13**: 423–41.

Kiran D, Podell KB, Chambers M, Basaraba JR (2016) Host-directed therapy targeting the *Mycobacterium tuberculosis* granuloma: a review. *Seminars in Immunopathology* **38**: 167–183.

Koczula MK, Gallotta A (2016) Lateral flow assays. *Essays in Biochemistry* **60**: 111–120.

Kolloli A, Subbian S (2017) Host-Directed Therapeutic Strategies for Tuberculosis. *Frontiers in Medicine* **4**: 171.

Köster S, Upadhyay S, Philips JA (2018) Why macrophages cannot LAP up TB. *Autophagy* **14**(3): 552–554.

Kryczek I, Zhao E, Liu Y, Wang Y, Vatan L, Szeliga W, Moyer J, Klimczak A, Lange A, Zou W (2011) Human TH17 cells are long-lived effector memory cells. *Science Translational Medicine* **3**: 104ra100, doi: 10.1126/scitranslmed.3002949.

Kumar SK, Singh P, Sinha S (2015) Naturally produced opsonizing antibodies restrict the survival of *Mycobacterium tuberculosis* in human macrophages by augmenting phagosome maturation. *Open Biology* **5**: 150–171.

Kupiec T (2004) Quality-Control Analytical Methods: High-Performance Liquid Chromatography. *International Journal of Pharmaceutical Compounding* **8**(3): 223–227.

Lagrange PH, Thangaraj SK, Dayal R, Despande A, Ganguly NK, Girardi E, Joshi B, Katoch K, Katoch VM, Kumar M, Lakshmi V, Leportier M, Longuet C, Malladi SV, Mukerjee D, Nair D, Raja A, Raman B, Rodrigues C, Sharma P, Singh A, Singh S, Sodha A, Kabeer BS, Vernet G, Goletti D (2012) A toolbox for tuberculosis diagnosis: an Indian multicentric study (2006-2008): microbiological results. *PLoS One* **7**: e43739.

Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, Mizushima N, Grinstein S, Iwasaki A (2010) *In vivo* requirement for Atg5 in antigen presentation by dendritic cells. *Immunity* **32**: 227–239.

Li Q, Singh CR, Ma S, Price ND, Jagannath C (2011) Label-free proteomics and systems biology analysis of mycobacterial phagosomes in dendritic cells and macrophages. *Journal of Proteome Research* **10**: 2425–2439.

Liu F, Chen J, Wang P, Li H, Zhou Y, Liu H, Liu Z, Zheng R, Wang L, Yang H, Cui Z, Wang F, Huang X, Wang J, Sha W, Xiao H, Ge B (2018) MicroRNA-27a controls the intracellular survival of *Mycobacterium tuberculosis* by regulating calcium-associated autophagy. *Nature Communications* doi: 10.1038/s41467-018-06836-4.

Löpez-Hernandez Y, Patino-Rodrıguez O, Garcıa-Orta ST, Pinos-Rodrıguez JM (2016) Mass spectrometry applied to the identification of *Mycobacterium tuberculosis* and biomarker discovery. *Journal of Applied Microbiology* **121**: 1485–1497.

Lu CM, Wu Y, Chen C-C, Hsu J-L, Chen J-L, Chen Y-FJ, Huang C-H, Ko Y-C (2011) Identification of low-abundance proteins via fractionation of the urine proteome with weak anion exchange chromatography. *Proteome Science* **9**: 17.

Luetkemeyer FA, Kendall AM, Wu X, Lourenço CM, Jentsch U, Swindells S, Qasba SS, Sanchez J, Havlir VD, Grinsztejn B, Sanne MI, Firnhaber C, Adult AIDS Clinical Trials Group A5255 Study Team (2014) Evaluation of Two Line Probe Assays for Rapid Detection of *Mycobacterium tuberculosis*, Tuberculosis (TB) Drug Resistance, and Non-TB Mycobacteria in HIV-Infected Individuals with Suspected TB. *Journal of Clinical Microbiology* **52**(4): 1052–1059.

Maertzdorf J, Repsilber D, Parida SK, Stanley K, Roberts T, Black G, Walzl G, Kaufmann SH (2011) Human gene expression profiles of susceptibility and resistance in tuberculosis. *Genes Immunity* **12**: 15–22.

Makhado AN, Matabane E, Faccin M, Pinçon C, Jouet A, Boutachkourt F, Goeminne L, Gaudin C, Maphalala G, Beckert P, Niemann S, Delvenne J, Delmée M, Razwiedani L, Nchabeleng M, Supply P, de Jong CB, André E (2018) Outbreak of multidrug-resistant tuberculosis in South

Africa undetected by WHO-endorsed commercial tests: an observational study. *Lancet Infectious Diseases* doi: http://dx.doi.org/10.1016/S1473-3099(18)30496-1.

Manga S, Perales R, Reaño M, D'Ambrosio L, Migliori BG, Amicosante M (2016) Performance of a lateral flow immunochromatography test for the rapid diagnosis of active tuberculosis in a large multicentre study in areas with different clinical settings and tuberculosis exposure levels. *Journal of Thoracic Disease* **8**(11): 3307–3313.

Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, Hengartner MO, Green DR (2011) Microtubule- associated protein 1 light chain 3 a (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proceedings of the National Academy of Sciences* **108**: 17396–17401.

Minion J, Leung E, Talbot E, Dheda K, Pai M, Menzies D (2011) Diagnosing tuberculosis with urine lipoarabinomannan: systematic review and meta-analysis. *European Respiratory Journal* **38**: 1398–405.

Mizushima N, Yoshimori T, Ohsumi Y (2011) The role of atg proteins in autophagosome formation. *Annual Reviews in Cell and Developmental Biology* **27**: 107–132.

Muranski P, Borman ZA, Kerkar SP, Klebanoff CA, Ji Y, Sanchez-Perez L, Sukumar M, Reger RN, Yu Z, Kern SJ, Roychoudhuri R, Ferreyra GA, Shen W, Durum SK, Feigenbaum L, Palmer DC, Antony PA, Chan CC, Laurence A, Danner RL, Gattinoni L, Restifo NP (2011) Th17 cells are long lived and retain a stem cell-like molecular signature. *Immunity* **35**: 972–85.

Ndiaye BP, Thienemann F, Ota M, Landry BS, Camara M, Dieye S, Esmail H, Goliath R, Huygen K, January V, Ndiaye I, Oni T, Raine M, Romano M, Satti I, Sutton S, Thiam A, Wilkinson KA, Mboup S, Wilkinson RJ, McShane H (2015) Safety, immunogenicity, and efficacy of the candidate tuberculosis vaccine MVA85A in healthy adults infected with HIV-1: a randomised, placebo-controlled, phase 2 trial. *Lancet Respiratory Medicine* **3**: 190–200.

Neuschlova M, Vladarova M, Kompanikova J, Sadlonova V, Novakova E (2017) Identification of Mycobacterium Species by MALDI-TOF Mass Spectrometry. *Advances in Experimental Medicine and Biology* **33**: 37–42.

Nurwidya F, Handayani D, Burhan E, Yunus F (2018) Molecular Diagnosis of Tuberculosis. *Chonnam Medical Journal* **54**(1): 1–9.

Ota OCM, Mendy FJ, Donkor S, Togun T, Daramy M, Gomez PM, Chegou NN, Sillah KA, Owolabi O, Kampmann B, Walzl G, Sutherland SJ (2014) Rapid diagnosis of tuberculosis using *ex vivo* host biomarkers in sputum. *European Respiratory Journal* **44**: 254–257.

Ouimet M, Koster S, Sakowski E, Ramkhelawon B, van Solingen C, Oldebeken S, Karunakaran D, Portal-Celhay C, Sheedy FJ, Ray TD, Cecchini K, Zamore PD, Rayner KJ, Marcel YL, Philips JA, Moore KJ (2016) *Mycobacterium tuberculosis* induces the miR-33 locus to reprogram autophagy and host lipid metabolism. *Nature Immunology* **17**: 677–86.

Pai M, Denkinger MC, Kik VS, Rangaka XM, Zwerling A, Oxlade O, Metcalfe ZJ, Cattamanchi A, Dowdy WD, Dheda K, Banaeih N (2014) *Clinical Microbiology Reviews* **3**: 20.

Perley CC, Frahm M, Click EM, Dobos KM, Ferrari G, Stout JE, Frothingham R (2014) The human antibody response to the surface of *Mycobacterium tuberculosis*. *PLoS One* **9**: e98938.

Petruccioli E, Petrone L, Vanini V, Cuzzi G, Navarra A, Gualano G, Palmieri F, Girardi E, Goletti D (2015) Assessment of CD27 expression as a tool for active and latent tuberculosis diagnosis. *Journal of Infection* **71**: 526–33.

Phillips M, Basa-Dalay V, Bothamley G, Cataneo NR, Lam KP, Natividad RPM, Schmitt P, Wai J (2010) Breath Biomarkers of Active Pulmonary Tuberculosis. *Tuberculosis (Edinb)* **90**(2): 145–51.

Pinhata WMJ, Cergole-Novella CM, Carmo SMA, Silva FRR, Ferrazoli L, Sacchi TC, de Oliveira SR (2015) Rapid detection of *Mycobacterium tuberculosis* complex by real-time PCR in sputum samples and its use in the routine diagnosis in a reference laboratory. *Journal of Medical Microbiology* **64**(9): 1040–1045.

Plotkin SA (2008) Vaccines: correlates of vaccine-induced immunity. *Clinical Infectious Diseases* **47**: 401–9.

Pooideh M, Jabbarzadeh I, Ranjbar R, Saifi M (2015) Molecular epidemiology of *Mycobacterium tuberculosis* isolates in 100 patients with tuberculosis using pulsed field gel electrophoresis. *Jundishapur Journal of Microbiology* **8**(7): e18274.

Prabowo AS, Zelmer A, Stockdale L, Ojha U, Smith SG, Seifert K, Fletcher HA (2019) Historical BCG vaccination combined with drug treatment enhances inhibition of mycobacterial growth ex vivo in human peripheral blood cells. *Nature scientific reports* **9**: 4842.

Rahman MMS, Maliha TU, Ahmed S, Kabir S, Khatun R, Shah AJ, Banu S (2018) Evaluation of Xpert MTB/RIF assay for detection of *Mycobacterium tuberculosis* in stool samples of adults with pulmonary tuberculosis. *PLoS One* **13**(9): e0203063.

Reddy RP, Raju N (2012) Gel Electrophoresis – Principles and Basics, Chapter 2. p. cm. ISBN 978-953-51-0458-2 Published by *InTech Janeza Trdine 9, 51000 Rijeka, Croatia.*

Rekha SR, Mily A, Sultana T, Haq A, Ahmed S, Kamal MSM, van Schadewijk A, Hiemstra SP, Gudmundsson HG, Agerberth B, Raqibet R (2018) Immune responses in the treatment of drug-sensitive pulmonary tuberculosis with phenylbutyrate and vitamin D3 as host directed therapy. *BMC Infectious Diseases* **18**: 303.

Richards DM, Endres RG (2014) The mechanism of phagocytosis: Two stages of engulfment. *Biophysical Journal* **107**(7): 1542–1553.

Riou C, Blas PP, Roberts L, Ronacher K, Walzl G, Manca C, Rustomjee R, Mthiyane T, Fallows D, Gray MC, Kaplan G (2012) Effect of standard tuberculosis treatment on plasma cytokine levels in patients with active pulmonary tuberculosis. *PLoS One* **7**: e36886.

Rodrigo G, Gruvegård M, Van Alstine MJ (2015) Antibody Fragments and Their Purification by Protein L Affinity Chromatography. *Antibodies* **4**(3): 259–277.

Rodriguez DC, Ocampo M, Salazar LM, Patarroyo MA (2018) Quantifying intracellular *Mycobacterium tuberculosis*: An essential issue for *in vitro* assays. *Microbiology Open* https://doi.org/10.1002/mbo3.588 accessed on 14th August 2019.

Sabir N, Hussain T, Shah SZA, Peramo A, Zhao D, Zhou X (2018) miRNAs in Tuberculosis: New Avenues for Diagnosis and Host-Directed Therapy. *Frontiers in Microbiology* **9**: 602.

Schiebler M, Brown K, Hegyi K, Newton SM, Renna M, Hepburn L, Klapholz C, Coulter S, Obregón-Henao A, Henao Tamayo M, Basaraba R, Kampmann B, Henry KM, Burgon J, Renshaw SA, Fleming A, Kay RR, Anderson KE, Hawkins PT, Ordway DJ, Rubinsztein DC, Floto RA (2015) Functional drug screening reveals anticonvulsants as enhancers of mTOR-independent autophagic killing of *Mycobacterium tuberculosis* through inositol depletion. *EMBO Molecular Medicine* **7**(2): 127–39.

Schon T, Lerm M, Stendahl O (2013) Shortening the 'short-course' therapy- insights into host immunity may contribute to new treatment strategies for tuberculosis. *Journal of internal medicine* **273**: 368–382.

Schroeder H, Cavacini L (2010) Structure and function of immunoglobulins. *Journal of Allergy and Clinical Immunology* **125**: S41–S52.

Sei JC, Shey B, Schuman FR, Rikhi N, Muema K, Rodriguez DJ, Daum TL, Fourie PB, Fischer WG (2019) Opsonic Monoclonal Antibodies Enhance Phagocytic Killing Activity and Clearance of *Mycobacterium tuberculosis* from Blood in a Quantitative qPCR Mouse Model. *Heliyon* **5**(9): 1–10.

Senoputra AM, Shiratori B, Hasibuan MF, Koesoemadinata CR, Apriani L, Ashino Y, Ono K, Oda T, Matsumoto M, Suzuki Y, Alisjahbana B, Hattori T (2015) Diagnostic value of antibody responses to multiple antigens from *Mycobacterium tuberculosis* in active and latent tuberculosis. *Diagnostic Microbiology and Infectious Disease* doi: http://dx.doi.org/10.1016/j.diagmicrobio.2015.07.021.

Seto S, Tsujimura K, Koide Y (2011) Rab GTPases regulating phagosome maturation are differentially recruited to mycobacterial phagosomes. *Traffic* **12**: 407–420.

Seto S, Tsujimura K, Horii T, Koide Y (2013) Autophagy Adaptor Protein p62/SQSTM1 and Autophagy-Related Gene Atg5 Mediate Autophagosome Formation in Response to *Mycobacterium tuberculosis* Infection in Dendritic Cells. *PLoS One* **8**(12): e86017.

Sharma A, Vaghasiya K, Guptab P, Guptab DU, Verma KR (2018) Reclaiming hijacked phagosomes: Hybrid nano-in-micro encapsulated MIAP peptide ensures host directed therapy by

specifically augmenting phagosome-maturation and apoptosis in TB infected macrophage cells. *International Journal of Pharmaceutics* **536**: 50–62.

Shekhawat DS, Jain KR, Gaherwar MH, Purohit JH, Taori MG, Daginawala FH, Kashyap SR (2014) Heat shock proteins: Possible biomarkers in pulmonary and extrapulmonary tuberculosis. *Human Immunology* **75**: 151–158.

Shin H-J, Franco LH, Nair VR, Collins AC, Shiloh MU (2017) A baculovirus-conjugated mimotope vaccine targeting *Mycobacterium tuberculosis* lipoarabinomannan. *PLoS One* **12** (10): e0185945.

Sigal GB, Segal MR, Mathew A, Jarlsberg L, Wang M, Barbero S, Small N, Haynesworth K, Davis JL, Weiner M, Whitworth WC, Jacobs J, Schorey J, Lewinsohn DM, Nahid P (2017) Biomarkers of tuberculosis severity and treatment effect: a directed screen of 70 host markers in a randomized clinical trial. *EBioMedicine* **25**: 112–121.

Singh SB, Ornatowski W, Vergne I, Naylor J, Delgado M, Roberts E, Ponpuak M, Master S, Pilli M, White E, Komatsu M, Deretic V (2010) Human IRGM regulates autophagy and cell-autonomous immunity functions through mitochondria. *Nature Cell Biology* **12**: 1154–65.

Singh B, Saqib M, Gupta A, Kumar P, Bhaskar S (2017) Autophagy induction by *Mycobacterium indicus pranii* promotes *Mycobacterium tuberculosis* clearance from RAW 264.7 macrophages. *PLoS One* **12**(12): e0189606.

Singh KB, Sharma KS, Sharma R, Sreenivas V, Myneedu PV, Kohli M, Bhasin D, Sarin S (2017) Diagnostic utility of a line probe assay for multidrug resistant-TB in smear-negative pulmonary tuberculosis. *PLoS One* **12**(8): e0182988.

Siqueira da Silva M, Ribeiro de Moraes R, Travassos HL (2018) Autophagy and its interaction with intracellular Bacterial Pathogens. *Frontiers in Immunology* **9**(935): 1–7.

Sotgiu G, Centis R, D'ambrosio L, Migliori BG (2015) Tuberculosis Treatment and Drug Regimens. *Cold Spring Harbor Perspectives in Medicine* **5**: a017822.

Sugaya K, Seto S, Tsujimura K, Koide Y (2011) Mobility of late endosomal and lysosomal markers on phagosomes analyzed by fluorescence recovery after photobleaching. *Biochemical and Biophysical Research Communications* **410**: 371–375.

Swanepoel CC, Snyders IC, Isaacs S, Abayomi AE, Grewal R (2015) Biomarker discovery for diagnosis and treatment of tuberculosis: a role for biobanking? *Journal of Biorepository Science for Applied Medicine* **3**: 47–56.

Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, Shea JE, Bruce BJ, Hussey GD, Hanekom WA, Mahomed H, McShane H (2013) Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* **381**: 1021–8.

Thabet S, Karboul A, Dekhil N, Mardassi H (2014) IS6110-5'3'FP: an automated typing approach for *Mycobacterium tuberculosis* complex strains simultaneously targeting and resolving IS6110 5' and 3' polymorphisms. *International Journal of Infectious Diseases* **29**: 211–218.

Tiberi S, Scardigli A, Centis R, D'Ambrosio L, Munoz-Torrico M, Salazar-Lezama AM, Spanevello A, Visca D, Zumla A, Migliori BG, Luna CAJ (2017) Classifying new anti-tuberculosis drugs: rationale and future perspectives. *International Journal of Infectious Diseases* **56**: 181–184.

Van Der Meeren O, Hatherill M, Nduba V, Wilkinson RJ, Muyoyeta M, Van Brakel E, Ayles HM, Henostroza G, Thienemann F, Scriba TJ, Diacon A, Blatner GL, Demoitie M, Tameris M, Malahleha M, Innes JC, Hellstrom E, Martinson N, Singh T, Akite EJ, Azam KA, Bollaerts A, Ginsberg AM, Evans TG, Gillard P, Tait DR (2018) Phase 2b Controlled Trial of M72/AS01E Vaccine to Prevent Tuberculosis. *New England Journal of Medicine* **379**: 1621–34.

Vilaplana C, Marzo E, Tapia G, Diaz J, Garcia V, Cardona PJ (2013) Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *Journal of Infectious Diseases* **208**: 199–202.

Wallis RS, Kim P, Cole S, Hanna D, Andrade BB, Maeurer M, Schito M, Zumla A (2013) Tuberculosis biomarkers discovery: developments, needs, and challenges. *Lancet Infectious Diseases* **13**(4): 362–372.

Walters E, Demers A-M, van der Zalm MM, Whitelaw A, Palmer M, Bosch C, Draper RH, Gie PR, Hesseling CA (2017) Stool Culture for Diagnosis of Pulmonary Tuberculosis in Children. *Journal of Clinical Microbiology* **55**: 3355–3365.

Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A (2011) Immunological biomarkers of tuberculosis. *Nature Reviews in Immunol*ogy **11**: 343–354.

Wang J, Zhu X, Xiong X, Ge P, Liu H, Ren N, Khan AF, Zhou X, Zhang L, Yuan X, Chen X, Chen Y, Hu C, Robertson DI, Chen H, Guo A (2018) Identification of potential urine proteins and microRNA biomarkers for the diagnosis of pulmonary tuberculosis patients. *Emerging Microbes and Infections* **7**: 63.

World Health Organization (2011) Commercial serodiagnostic tests for diagnosis of tuberculosis: policy statement, 2011.

World Health Organization (2018) *Global tuberculosis report 2018* Geneva. Licence: CC BY-NC-SA 3.0 IGO.

Yerlikaya S, Brogera T, MacLean E, Paib M, Denkinger MC (2017) A tuberculosis biomarker database: the key to novel TB diagnostics. *International Journal of Infectious Diseases* **56**: 253–257.

Young LB, Mlamla Z, Gqamana PP, Smit S, Roberts T, Peter J, Theron G, Govender U, Dheda K, Blackburn J (2014) The identification of tuberculosis biomarkers in human urine samples. *European Respiratory Journal* **43**: 1719–1729.

Zhang C, Yang L, Zhao N, Zhao Y, Shi C (2018) Insights into Macrophage Autophagy in Latent Tuberculosis Infection: Role of Heat Shock Protein 16.3. *DNA and Cell Biology* **37**(5).

Ziegenbalg A, Prados-Rosales R, Jenny-Avital ER, Kim RS, Casadevall A, Achkar JM (2013) Immunogenicity of mycobacterial vesicles in humans: identification of a new tuberculosis antibody biomarker. *Tuberculosis Edinb* **93**: 448–55.

Zimmermann N, Thormann V, Hu B, Köhler A, Imai-Matsushima A, Locht C, Arnett E, Schlesinger SL, Zoller T, Schürmann M, Kaufmann HES, Wardemann H (2016) Human isotype-dependent inhibitory antibody responses against *Mycobacterium tuberculosis*. *EMBO Molecular Medicine* **8**: 1325–1339.

Zumla A, Rao M, Wallis SR, Kaufmann HES, Rustomjee R, Mwaba P, Vilaplana C, Yeboah-Manu D, Chakaya J, Ippolito G, Azhar E, Hoelscher M, Maeurer M, for the Host-Directed Therapies Network consortium (2016) Host-directed therapies for infectious diseases: current status, recent progress, and future prospects. *Lancet Infectious Diseases* **16:** e47–63.

Chapter 3

3 Opsonic monoclonal antibodies enhance phagocytic killing activity and clearance of *Mycobacterium tuberculosis* from blood in a quantitative PCR mouse model

3.1 Overview

Summary: Opsonic monoclonal antibodies (MABs) directed against *Mycobacterium tuberculosis* (MTB) that enhance phagocytic killing activity may be useful to enhance tuberculosis (TB) immunity. This chapter presents data to show that two novel monoclonal antibodies (GG9 and JG7) bound to killed and live *Mycobacterium smegmatis* (SMEG) and MTB (susceptible and resistant) and promoted opsonophagocytic killing activity (OPKA) against live MTB.

Contribution to the thesis and novelty: Work is presented on MAB-binding to various *M. tuberculosis* (susceptible, multi-drug resistant and extensively drug resistant) strains, from experiments conducted as part of a proof-of-concept study, alongside preliminary findings on opsonophagocytosis of live susceptible *M. tuberculosis* by a human macrophage cell line.

Contributions of Candidate: All work involving live MTB has been conducted, analysed and interpreted by the candidate at UP, which serves as definitive proof of the concept in line with the stated objectives of the overall thesis.

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3.2 Abstract

3.2.1 Background

Patients with impaired immunity often have rapid progression of tuberculosis (TB) which can lead to highly lethal *Mycobacterium tuberculosis* (MTB) sepsis. Opsonic monoclonal antibodies (MABs) directed against MTB that enhance phagocytic killing activity and clearance of MTB from blood may be useful to enhance TB immunity.

3.2.2 Methods

BALB/c mice were immunized with ethanol-killed MTB (EK-MTB) and MABs were produced and screened by ELISA for binding to killed and live *Mycobacterium smegmatis* (SMEG) and MTB. MAB opsonophagocytic killing activity (OPKA) was examined using SMEG with HL60 and U-937 cells and MTB with U-937 cells. Clearance of MTB from blood was evaluated in Institute of Cancer Research (ICR) mice given opsonic anti-MTB MABs or saline (control) 24 h prior to intravenous infusion with 10^8 CFUs gamma-irradiated MTB (HN878). MTB levels in murine blood collected 0.25, 4 and 24 h post-challenge was assessed by qPCR. MAB binding to peptidoglycan (PGN) was examined by ELISA using PGN cell wall mixture and ultra-pure PGN.

3.2.3 Results

Two MABs (GG9 and JG7) bound to killed and live SMEG and MTB (susceptible and resistant) and promoted OPKA with live MTB. MAB JG7 significantly enhanced OPKA of MTB. Both MABs significantly enhanced clearance of killed MTB from murine blood at 4 and 24 h as measured by qPCR. These opsonic MABs bound to PGN, a major cell wall constituent.

3.2.4 Conclusions

Anti-MTB MABs that promote bactericidal phagocytic activity of MTB and enhance clearance of killed MTB from the blood, may offer an immunotherapeutic approach for treatment of MTB bacteraemia or sepsis, and augment treatment of multi-drug resistant (MDR) or extensively drug resistant (XDR) TB.

3.3 Manuscript

A copy of the full paper as it appeared in *Heliyon* is included below.

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Opsonic monoclonal antibodies enhance phagocytic killing activity and clearance of *Mycobacterium tuberculosis* from blood in a quantitative qPCR mouse model

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ABSTRACT

Background: Patients with impaired immunity often have rapid progression of tuberculosis (TB) which can lead to highly lethal *Mycobacterium tuberculosis* (MTB) sepsis. Opsonic monoclonal antibodies (MABs) directed against MTB that enhance phagocytic killing activity and clearance of MTB from blood may be useful to enhance TB immunity.

Methods: BALB/c mice were immunized with ethanol-killed MTB (EK-MTB) and MABs were produced and screened by ELISA for binding to killed and live *Mycobacterium smegmatis* (SMEG) and MTB. MAB opsonophagocytic killing activity (OPKA) was examined using SMEG with HL60 and U-937 cells and MTB with U-937 cells. Clearance of MTB from blood was evaluated in Institute of Cancer Research (ICR) mice given opsonic anti-MTB MABs or saline (control) 24 h prior to intravenous infusion with 10^8 CFUs gamma-irradiated MTB (HN878). MTB levels in murine blood collected 0.25, 4 and 24 h post-challenge were assessed by qPCR. MAB binding to peptidoglycan (PGN) was examined by ELISA using PGN cell wall mixture and ultra-pure PGN.

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Conclusions: Anti-MTB MABs that promote bactericidal phagocytic activity of MTB and enhance clearance of killed MTB from the blood, may offer an immunotherapeutic approach for treatment of MTB bacteremia or sepsis, and augment treatment of multi-drug resistant (MDR) or extensively drug resistant (XDR) TB.

1. Introduction

Tuberculosis (TB) ranks among the top ten causes of death worldwide and continues to be a major threat to global health [1]. In 2017, there were an estimated 10 million new TB cases worldwide, of which 1.6 million individuals died of the disease [1, 2]. Additionally, an estimated 0.9 million of these new cases were HIV-positive of which 0.3 million (30%) died of TB. In sub-Saharan Africa, many HIV positive individuals are co-infected with TB [2, 3]. In HIV-positive patients, TB is the leading cause of death [2], and many develop MTB bacteremia, sepsis and disseminated disease [4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15], often dying within 30 days of hospitalization [10, 15]. Furthermore, strains of MTB are becoming increasingly resistant to antibiotic therapy, and the emergence of multiple drug resistant (MDR) and extensively drug resistant (XDR) strains have increased the urgency to find new approaches for prevention and treatment of TB [16].

In the pre-antibiotic period, specific antibacterial serum was deemed beneficial in the treatment of pneumococcal pneumonia and sepsis [17, 18]. The development of antibiotics decreased the use of anti-serum therapy until the availability of intravenous immune globulin (IVIG) in the 1980's [19, 20]. Antibodies in IVIG that bound to the bacterial capsule of gram positive bacteria enhanced phagocytosis and provided protection

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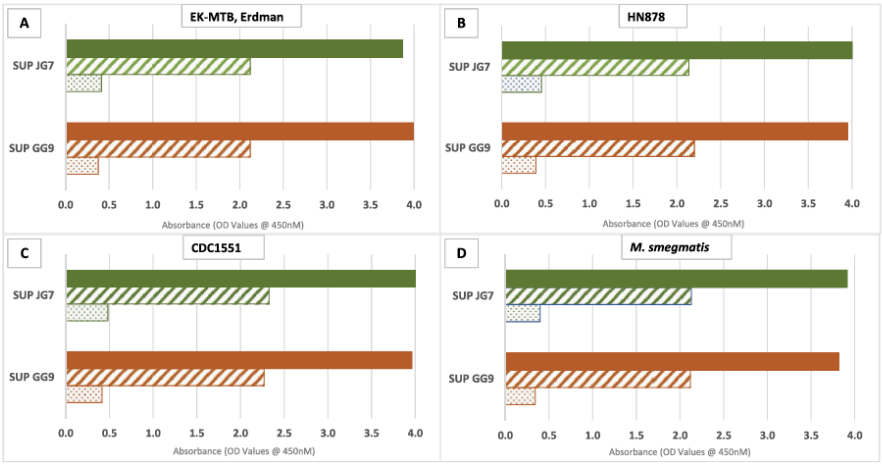

Fig. 1. Binding activities of supernatants from hybridomas JG7and GG9 to *Mycobacterium tuberculosis* (MTB) and *Mycobacterium smegmatis* (SMEG), evaluated at dilutions 1:10 (solid), 1:100 (diagonal stripes) and 1:1000 (dotted) on fixed mycobacteria at 1×10^5 CFU/well. Panel (A) through (C), respectively, shows binding of supernatant to killed MTB Erdman, HN878 and CDC1551. Panel (D) depicts binding of supernatants to fixed SMEG. OD values for growth media without antibody (negative control) range between 0.046–0.060 (data not shown).

in vivo [19, 20]. In addition, the ability to produce monoclonal antibodies (MABs) that react to specific pathogens and are functionally active has provided highly effective therapeutics with minimal side effects [21]. MTB has a thick waxy capsule which is an important virulence factor enabling survival in the infected host [22, 23, 24, 25, 26] and anti-capsular antibodies may promote phagocytosis and killing of MTB [24]. During infection, MTB bacilli are engulfed by macrophages which subsequently enter phagosomes [27]. Membrane-bound proteins on the mycobacteria-containing phagosomes recruit lysosomes leading to phagosome-lysosome fusion (P-L fusion) forming mature phagolysosomes which kill and digest MTB [28]. Conversely, MTB evades this defense mechanism by persisting in macrophages [29]. Antibodies to MTB have been shown to promote FcγR-mediated macrophage phagocytosis, phagolysosome maturation and MTB killing and therefore may play an important role in phagocytic killing of MTB [30]. While evidence suggests that cell-mediated immunity is the protective immune function against MTB [31], the emergence of MDR and XDR MTB strains coupled with inadequate protection (among adults) from current vaccine strategies [31] has led to a renewed interest in exploring the potential use of antibodies to prevent or treat TB [30]. Evidence suggests that antibodies could play an important role in intracellular killing of MTB [32] and recent studies have demonstrated that opsonic antibodies promote phagolysosome maturation that enhances phagocytic killing of MTB [24, 28, 32].

In this report, we describe novel MABs directed against MTB that promote phagocytosis and killing of mycobacteria (MTB and SMEG) as well as enhance clearance of MTB DNA from the blood in a murine MTB bacteremia model.

2. Materials and methods

2.1. Mycobacterium smegmatis (SMEG)

M. smegmatis ATCC Cat 21701 (ATCC, Manassas, VA, USA) was cultured aerobically in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) overnight to mid logarithmic phase at 37°C with shaking at 250rpm and the resulting suspension used as a surrogate for MTB.

2.2. Mycobacterium tuberculosis (MTB), inactivated laboratory isolates

Ethanol-killed MTB (EK-MTB), strain Erdman, ATCC 35801 (Battelle, Columbus, OH, USA) was provided at 10^8 CFU/mL (OD 600nM = 1.000). Gamma Irradiated MTB, strains HN878 (NR-14821) and CDC1551 (NR-14820) were obtained from BEI Resources (Manassas, VA, USA) at 8.7×10^{10} CFU/mL.

2.3. Mycobacterium tuberculosis (MTB), live laboratory and clinical isolates

H37Ra ATCC 25177, two susceptible clinical MTB strains (STB1 and STB2), three multidrug-resistant MTB strains (MDR1, MDR2 and MDR3) and two extensively drug-resistant MTB strains (XDR1, XDR2) were used. Strains were obtained from both the National Health Laboratory Service-Tshwane Academic Division (NHLS/TAD) in Pretoria and the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. The standard Ziehl-Neelson acid-fast staining technique was carried out on the cultures and viewed under the microscope for the presence of mycobacteria and the MPT64TB (SD Bioline, South Korea) antigen test was carried out for confirmation of MTB.

2.4. Mice immunizations

Female BALB/c mice 3-4-week-old, Harlan Laboratories (Indianapolis, IN, USA) were obtained for use in this study. Stock EK-MTB (vaccine) was centrifuged at 12000rpm for 5 min at room temperature (RT), washed three times in Phosphate Buffered Saline pH 7.4 (PBS, Fisher Scientific, Pittsburg, PA, USA) and 100μL of the immunogen injected subcutaneously, without adjuvant, into mice on Day 0 at approximately 1×10^5 CFU/mouse. Boosts were given per two protocols: (a) days 7, 16, 27 and 42 with blood samples obtained prior to initiation of the first protocol, and on days 19, 41, and 63 for Mice 1319-1324, and (b) days 14, 29 and 43 with blood samples obtained prior to the initiation of the second protocol and on days 28, 35, 42 and 63 for Mice 1417-1420. Three days prior to fusion, mice were given an intravenous final boost of 1×10^4 bacteria. About 150–200 μL of blood was collected at each bleed. All animal procedures were performed in an AALAC-accredited facility. All procedures were reviewed and approved by the facility IACUC.

2.5. Fusion and hybridoma production

Two BALB/c mice (Mice 1323 and 1420 – one from each

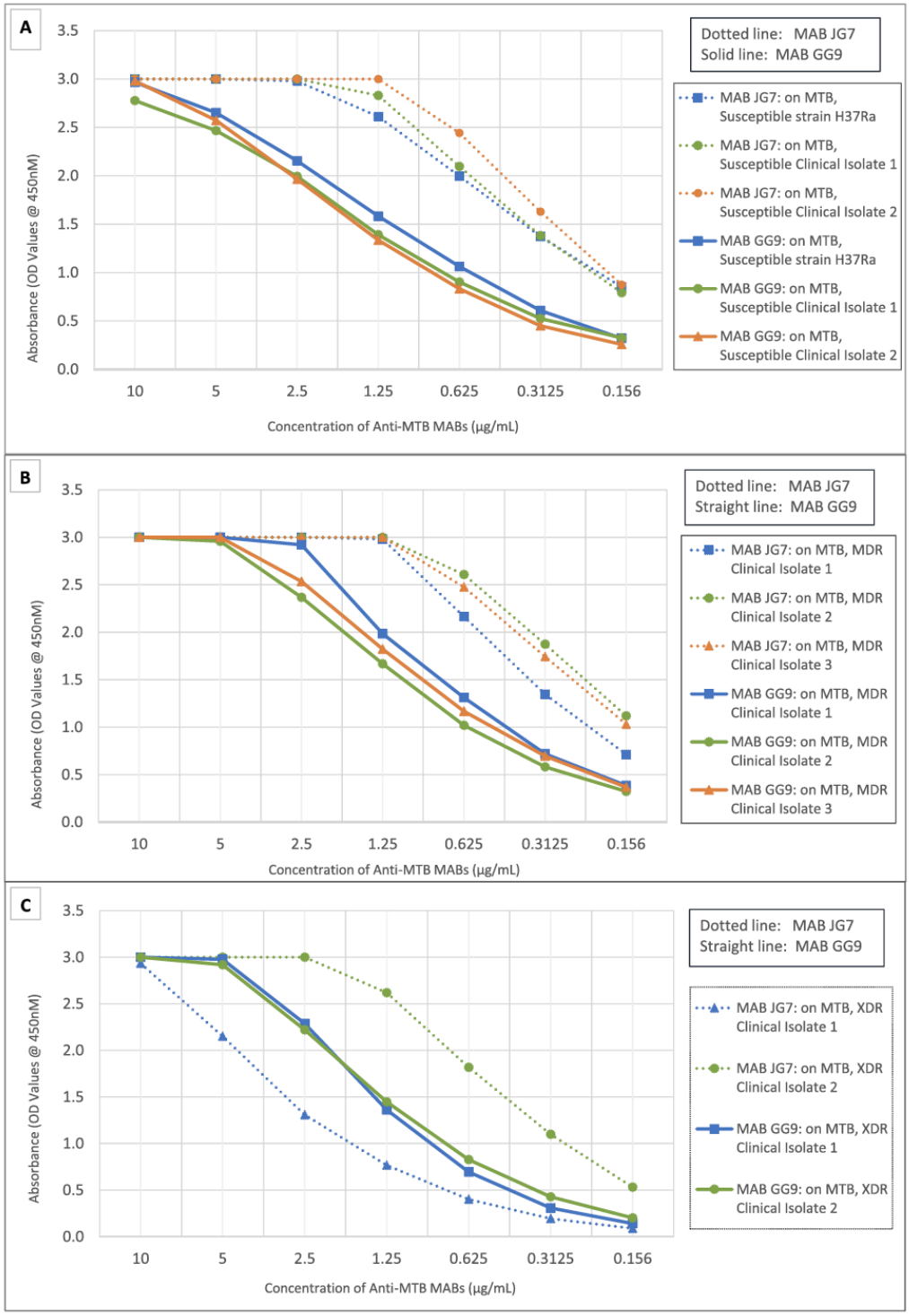

Fig. 2. Binding activity of purified anti-*Mycobacterium tuberculosis* monoclonal antibodies (anti-MTB MABs) JG7 and GG9 on fixed MTB at 1×10^5 CFU/well. Panel (A) demonstrates MAB binding to susceptible lab strain H37Ra and clinical isolates 1 & 2; Panel (B) to multidrug-resistant (MDR) clinical isolates 1, 2 & 3; and Panel (C) to extensively drug-resistant (XDR) clinical isolates 1 and 2. Data (expressed as mean) are representative of three individual experiments.

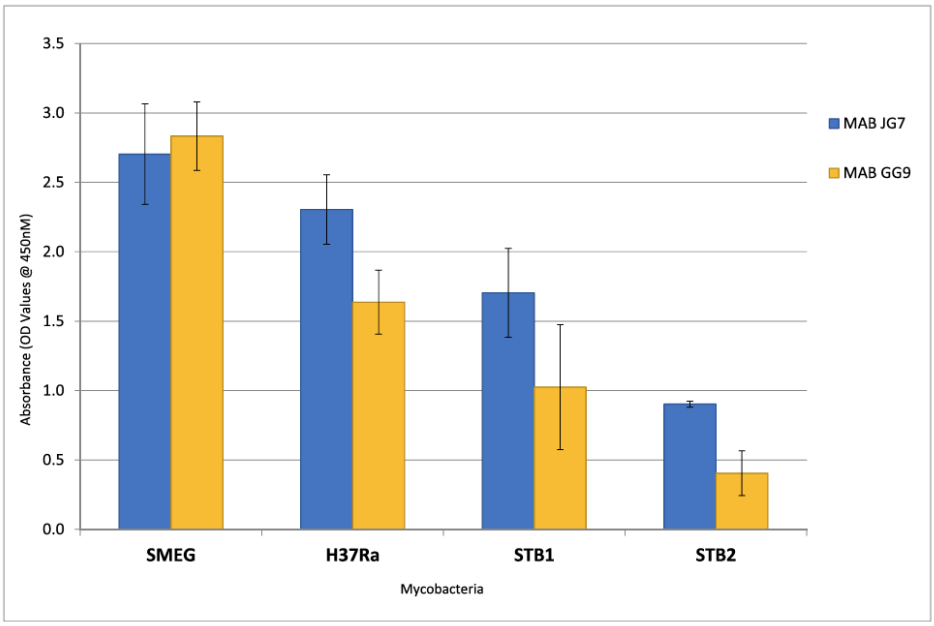

Fig. 3. Binding activity of purified anti-*Mycobacterium tuberculosis* monoclonal antibodies (anti-MTB MABs) GG9 and JG7 to live *Mycobacterium smegmatis* (SMEG) and live susceptible MTB H37Ra (lab strain) and STB1 and STB2 (susceptible clinical isolates) as demonstrated in a Live Bacteria ELISA. Data (expressed as mean ± standard errors; n = 3) are representative of three individual experiments.

immunization protocol) with high anti-MTB serum titers were euthanized [33], and single cell spleen suspensions from each mouse [34] were fused to SP2/0 myeloma cells (Sigma-Aldrich, St. Louis, MO, USA) using standard techniques [34, 35]. Hybridoma supernatants were tested by Enzyme-Linked Immunosorbent Assay (ELISA) for binding to EK-MTB and high producing cells were selected for cloning, tested further for MAB production, and then expanded for supernatant collection, purification by Protein G Column Chromatography [36] and quantification by IgG Capture ELISA [35].

2.6. Detection of antibodies that bind to mycobacteria

2.6.1. Anti-sera ELISA

Serum anti-MTB levels were evaluated using killed MTB. 96-well NUNC™ MaxiSorp ELISA plates (Fisher Scientific, Pittsburg, PA, USA) were coated with EK-MTB in 70% alcohol (Fisher Scientific, Pittsburg, PA, USA) at 100μL per well (1×10^5 CFU) for 18–24 h at room temperature (RT) or up to 2 days at 2–8°C. MTB-coated plates were blocked with 3% Normal Goat Serum (NGS, Southern Biotech, Birmingham, AL, USA) in PBS and subsequently, washed with PBS-0.05% Tween 20 (PBS-T, Fisher Scientific, Pittsburg, PA, USA) using an ELx405 Automated Plate Washer (BioTek, Winooski, VT, USA). Serial dilutions of serum samples were added. Anti-MTB antibodies were detected with Horse Radish Peroxidase (HRP)-Conjugated, gamma-specific, Goat anti-Mouse IgG (Southern Biotech, Birmingham, AL, USA) or HRP-Conjugated, IgG isotype-specific Goat anti-Mouse IgG1, IgG2a, IgG2b, IgG3 (Southern Biotech, Birmingham, AL, USA). TMB Substrate Solution (Fisher Scientific, Pittsburg, PA, USA) was added prior to being quenched with TMB STOP solution (Fisher Scientific, Pittsburg, PA, USA). The absorbance values (450nM) of each well were obtained using a SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Pre-immunization sera were used as negative controls.

2.6.2. Fixed ELISA

Hybridoma supernatants were screened for their capacity to bind to killed laboratory MTB isolates: Ethanol-killed Erdman, and Gamma-irradiated HN878 and CDC1551 MTB strains, using procedures described above, excluding the blocking step with 3% NGS.

Purified anti-MTB MABs were screened for their capacity to bind to clinical susceptible, MDR and XDR isolates. MTB suspensions, from either ATCC or BACTEC 960-mycobacterial growth incubation tube (MGIT) stock cultures, were sub-cultured in MGITs at 1:10 dilutions (0.5mL in 5mL) containing Middlebrook 7H9 broth and incubated in the BACTEC 960 machine at 37°C for approximately three weeks, until an optical density (OD600nM) of 0.120–0.150 was reached. The OD was measured using a HELIOS spectrophotometer (Thomas Scientific, Swedesboro, NJ, USA). The MTB strains were then washed three times in PBS (pH 7.2–7.4, tablets from Sigma-Aldrich Life Science, Chemie GmBH) by adding 3mL of the bacterial suspension to 15mL of PBS (1:5 dilution), and centrifuged at 2600rpm for 5 min at RT. The washed bacteria were resuspended in 3mL of PBS (1mL per 96-well plate, per MAB). 96-well NUNC™ MaxiSorp flat-bottom ELISA plates (Sigma-Aldrich life science, Chemie GmbH) were coated with serial dilutions of MTB at 100μL per well and incubated for at least 18 h at RT. The MTB coated plates were washed using an automated plate washer (Biotek, Winooski, VT, USA). Serial dilutions of the MABs were added and incubated for 1 h at RT. The anti-MTB MABs were then detected with IgG1 detection antibody (Southern Biotechnologies, Birmingham, AL, USA). TMB substrate solution (Sigma-Aldrich life science, Chemie GmbH) was added and after 15 min the reaction was quenched with TMB STOP solution (Sigma-Aldrich life science, Chemie GmbH). The absorbance was read at 450nM using the ELx800 reader (Biotek, Winooski, VT, USA).

2.6.3. Live bacteria ELISA for MAB-binding to SMEG

The ability of purified anti-MTB MABs to bind to live SMEG was

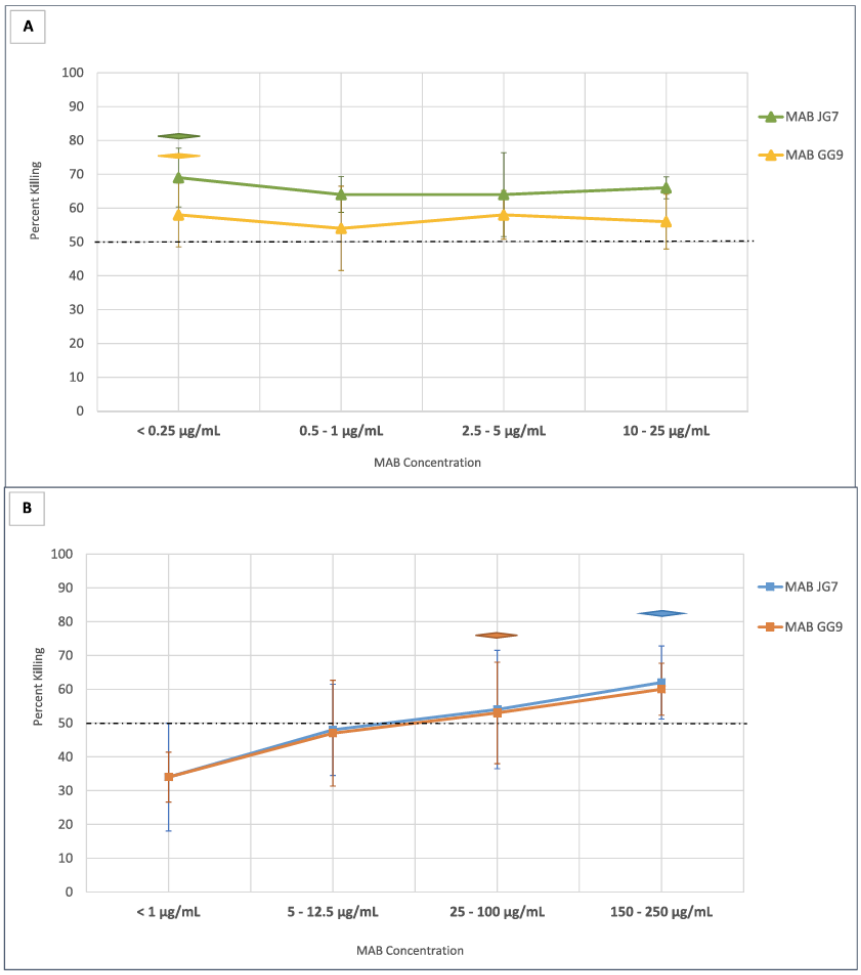

Fig. 4. Enhanced OPKA of MABs JG7 and GG9 against *Mycobacterium smegmatis* (SMEG) using HL60 granulocytes and C1q (Panel A) occurred at low antibody concentrations (< 0.25µg/mL) and stayed constant when antibody levels were increased over one hundred fold. While MAB JG7 consistently had higher percent killing, the difference did not reach statistical significance. Peak OPKA for both JG7 and GG9 occurred at 0.06µg/mL and were 81% (green icon) and 76% (yellow icon), respectively. In panel B, enhanced MAB OPKA against SMEG using U-937 macrophages (without C1q) was significantly more pronounced at higher antibody concentrations (JG7: $p = 0.0001$, GG9: $p < 0.0001$) and both MABs tracked closely together across all antibody concentrations. Peak OPKA for JG7 and GG9 were 82% (blue icon) at 175µg/mL and 76% (orange icon) at 100µg/mL), respectively.

evaluated to confirm use of SMEG as target mycobacteria in opsonic assays. Overnight culture at mid-log phase was adjusted to 40% transmittance and washed in PBS containing 0.1% BSA (Sigma-Aldrich, St. Louis, MO, USA) by centrifugation at 2500rpm for 5 min at RT to remove traces of 7H9 broth, resuspended in PBS with 0.1% BSA and added to round-bottom 96-well plates (VWR, Randor, PA, USA) at 100µL per well, (~10^6 CFU/well). 50µL of diluted MABs (1, 10 and 25 µg/mL) were added on top of SMEG and placed in a 37°C shaker incubator at 100rpm for 1 h. Subsequently, assay plates containing bacteria and MAB were washed three times by centrifugation. After washing, HRP-conjugated isotype-specific goat anti-mouse IgG (Southern Biotechnologies, Birmingham, AL, USA) was added with subsequent incubation at RT for 30 min. The assay plates were washed and TMB Substrate Solution was added prior to quenching with TMB STOP Solution. The assay plates were subsequently centrifuged to pellet the bacteria and 180µL of each sample was then carefully transferred into a NUNC™ Maxisorp 96-well ELISA plate, without disturbing the bacterial pellet, and absorbance at 450nM was determined using a SpectraMax Plus Microplate Reader.

2.6.4. *Live bacteria ELISA for MAB-binding to live MTB*

The ability of purified anti-MTB MAB JG7 to bind to live MTB was evaluated. The same bacterial ODs that were used for the fixed assay were used for the live Bacteria ELISAs to confirm binding to live MTB prior to beginning opsonic studies. MTB strains, H37Ra and clinical susceptible MTB, were grown to mid-logarithmic phase and serially diluted in PBS/BSA solution. The dilutions were dispensed into 96-well polypropylene plates (Sigma-Aldrich life science, Chemie GmbH) in 100µL volumes. Various concentrations of JG7 were made from stock, in PBS-T and 50µL of each resulting concentration was dispensed into each well of the plates containing bacteria. The plates were sealed and incubated for 1 h at 37°C in a shaking incubator (Thermostar, BMG Labtech, Ortenberg, Germany) at 250rpm.

After incubation, the plates were centrifuged at 2600rpm for 5–10 min at 25°C. The plates were manually washed twice with PBST/BSA. A 100µL volume of diluted goat anti-mouse IgG1 detection antibody was dispensed into each well of the plates, sealed and incubated in the shaking incubator at 37°C for 30 min at 250rpm. The plates were washed thrice with PBS-T/BSA. After the final wash, the solution was pipetted out completely and 100µL of TMB substrate solution was dispensed into the wells. The plate was incubated in the dark for 15 min at room temperature. One hundred microliters (100µL) of TMB stop solution was added into the wells after the incubation and centrifuged at 2600rpm for

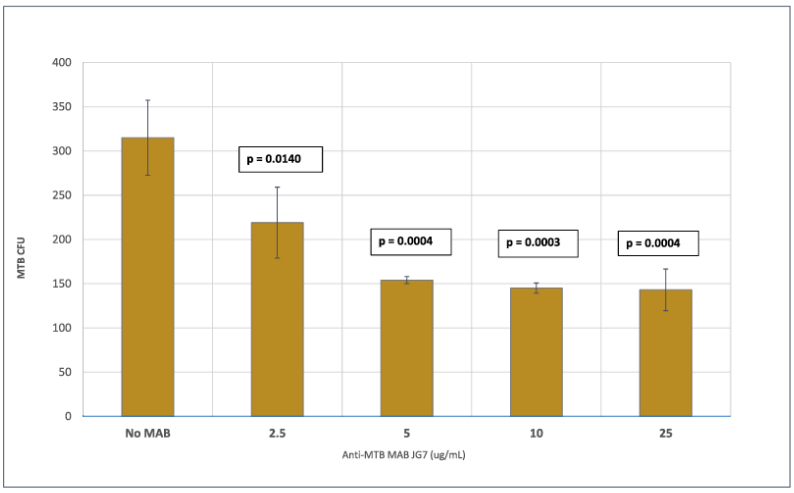

Fig. 5. OPKA of MAB JG7 against live *Mycobacterium tuberculosis* (MTB) clinical isolate STB1, using U-937 macrophages (without C1q) was significantly enhanced at MAB levels 2.5–25 μg/mL. Compared to the control sample wells (without MAB), antibody sample wells had CFU counts that were significantly reduced ($p < 0.5$) from 315 (No MAB) to 219 (2.5μg/mL), 154 (5μg/mL), 145 (10μg/mL) and 143 (25μg/mL).

5 min at 25°C. A 180μL volume of the supernatant was carefully taken out (without the pellet) per well, and transferred onto corresponding wells of the ELISA NUNC™ Maxisorp flat-bottom plate. The NUNC™ plate was read immediately at either 450nM or 630nM, depending on the TMB STOP solution used.

2.7. Opsonophagocytic killing activity (OPKA)

Two novel anti-MTB MABs were selected for evaluation of their opsonophagocytic activity (OPKA) against SMEG using two effector cell lines: using HL60 cells differentiated to granulocytes and U-937 cells differentiated to macrophages, with MAB concentration ranges of 0.06–25 μg/mL and 0.05–250 μg/mL, respectively. Notably, anti-MTB MAB OPKA against MTB was examined using the U-937 macrophage cell line only.

2.7.1. Granulocytic cell line (against SMEG)

Human leukemia promyelocytic (HL60) cells (Sigma-Aldrich, St. Louis, MO and ATCC, Manassas, VA, USA) were cultured in complete growth medium consisting of RPMI-1640, 10% heat-inactivated fetal bovine serum (FBS; Fisher Scientific, Pittsburg, PA, USA) and 2mM L-glutamine (Fisher Scientific, Pittsburg, PA, USA). Cells were differentiated into granulocytes, using 1.25% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) for five to six days and subsequently diluted to 5×10^7 cells/mL in OP assay medium consisting of DMEM-F12 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10mM HEPES (Sigma-Aldrich, St. Louis, MO, USA). Sextuplet reaction wells were prepared by sequential addition of: 40μL diluted MAB, 40μL differentiated HL60s at 2×10^6 cells/well (5×10^7 cells/mL), 10μL diluted complement component C1q (VWR, Radnor, PA, USA), and 10μL of SMEG at 1–5x10^4 CFU/mL. The reaction mixture was incubated at 37°C for 4 h in a Max@ 4450 Orbital Shaker (Fisher Scientific, Pittsburg, PA, USA) at 300rpm. Following incubation, HL60 cells were lysed by transferring 10μL from the reaction wells into 190μL of ice cold tissue culture grade water (Fisher Scientific, Pittsburg, PA, USA) containing 0.1% BSA, then incubated on ice at 2–6°C for 30 min. A 100μL aliquot of each sample was plated on 5% sheep blood agar (Fisher Scientific, Pittsburg, PA, USA), incubated for 46–48 h at 37°C, and subsequently enumerated for colony forming units (CFU) using a MiniCount colony counter (IPI, Simi Valley, CA, USA). Bacterial killing activity was defined as the percentage of mean CFU counts in sample wells (with MAB, HL60 and C1q) divided by mean CFU counts in control wells (with HL60 and C1q but without MAB). HL60 cell opsonic activity and bactericidal killing has been previously considered antibody enhanced when the percentage of SMEG killed in the presence of MAB and C1q was ≥50% compared to values from HL60 cells and C1q alone [37]. Three independent HL60 cell opsonic assays were analyzed and OPKA across a range of concentrations was plotted.

2.7.2. Macrophage cell line (against SMEG)

Human histiocytic lymphoma monocytic (U-937) cells (ATCC, Manassas, VA, USA) were seeded into flat bottom tissue culture plates (Fisher Scientific, Pittsburg, PA, USA) at 1×10^6 cells/mL and differentiated for 3 days using 50ng/mL of Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) in complete growth media. The medium was changed after 24 h to complete growth medium - without PMA. U-937 cells grow as a suspension culture of monocytic cells prior to differentiation and become adherent macrophages-like post-differentiation. After differentiation, complete growth medium was replaced with 50μL per well of pre-warmed OP assay medium. Octuplet reaction wells were prepared by addition of 40μL diluted MAB directly into the wells with U-937 cells followed by 10μL of SMEG or MTB at 1×10^5 CFU/well. In previous bacterial studies, complement did not increase U-937 cell activity. The reaction mixture was incubated for 4 h at 37°C with shaking at 300rpm in a Max@ 4450 Orbital Shaker. After incubation, the entire 100μL of reaction mixture in each well was carefully discarded to remove extracellular bacteria. A wash with 200μL of PBS was performed and adherent cells were then lysed to release intracellular bacteria by adding ice cold tissue culture grade water containing 0.1% BSA and incubated on ice for 30 min. Each sample was plated on 5% sheep blood agar, incubated for 46–48 h at 37°C, and CFUs counted. The OP killing data was defined as the percentage of mean CFU counts in sample wells, divided by the mean CFU counts in controls wells, containing no MAB. Three independent U-937 opsonic assays were analyzed and OPKA across a range of concentrations was plotted.

2.7.3. Macrophage cell line (against MTB)

U-937 cells were differentiated same as the method described above for SMEG. Pure cultures of MTB (H37Ra ATCC 25177 and clinical

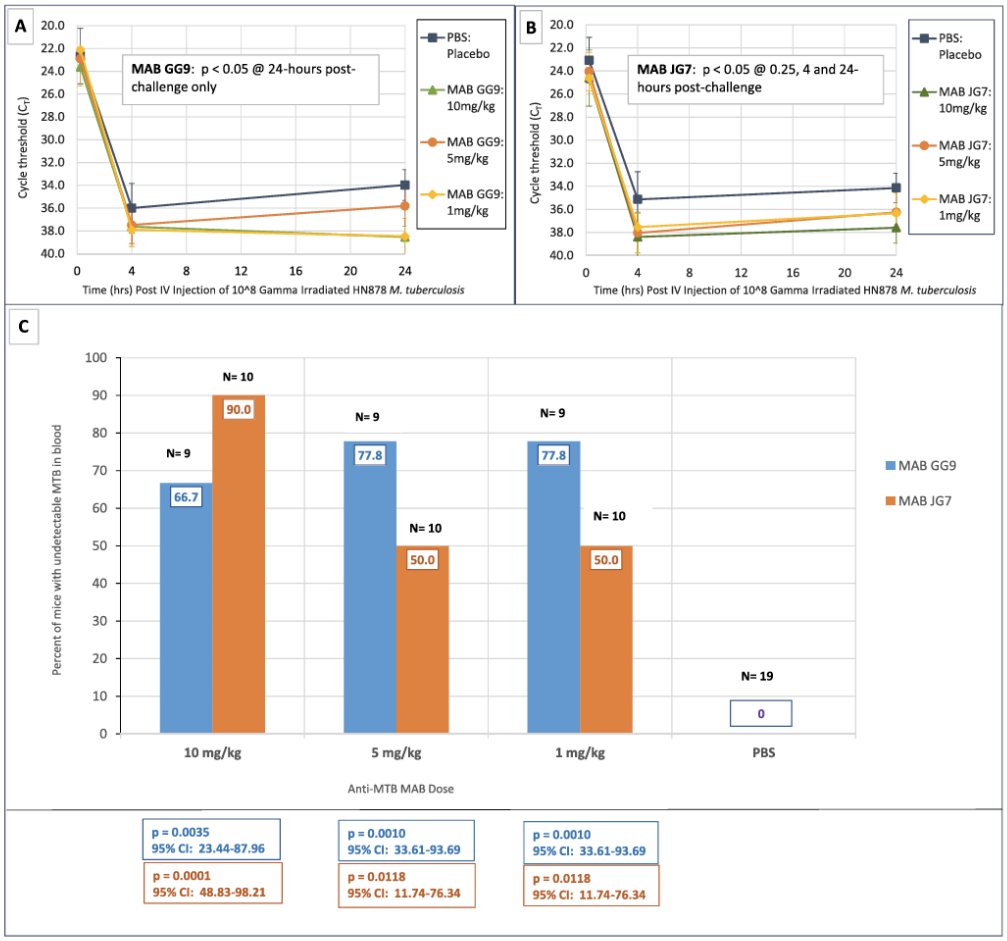

Fig. 6. Using qPCR, rapid clearance of *Mycobacterium tuberculosis* (MTB) in blood was observed in all groups from the *in vivo* study with N = 76 ICR mice. While MAB GG9 (panel A) significantly enhanced blood clearance at 24 h post challenge (1 mg/kg p = 0.0021, 10 mg/kg p = 0.0013), MAB JG7 (panel B) significantly enhanced clearance at all time points (0.25, 4 and 24 h) and at one or more doses. Panel C shows the percentage of mice with undetectable levels of MTB in blood according to qPCR. Statistical significance determined by comparison of MAB-treated vs. PBS-treated blood samples from mice according to no detection (i.e. $C_T = 40$, qPCR) was calculated using the Chi-squared test, with significance threshold set at $p < 0.05$ and 95% confidence intervals shown.

susceptible TB) were grown in MGIT 7H9 broth at 37°C until mid-logarithmic phase (1×10^6 CFU/mL – 1×10^8 CFU/mL). Two bacterial dilutions were used in each assay run (1:100 and 1:1000) in order to assess any differences in phagocytosis based on bacterial load. JG7 was used at concentrations ranging from 0.25–100 μg/mL. A volume of 40μL of JG7, followed by 10μL of either of the two dilutions of MTB was added on top of 50μL of the adherent, differentiated U-937 cells in sextuplet wells per sample. The plate was sealed and incubated for 4 h at 37°C with shaking at 300rpm in an orbital shaker (Thermostar, BMG Labtech, Ortenberg, Germany). After incubation, 90μL of the reaction mixture was discarded from each well on the incubation plate and 190μL of ice cold 0.1% BSA (30% BSA, Sigma-Aldrich Life Science, Chemie GmBH), diluted in sterile tissue culture grade water (Sigma-Aldrich life science, Chemie GmBH) was added to each well to lyse the cells. The plate was incubated on ice for 30 min. 100μL of each sample was plated on Middlebrook 7H10 or 7H11 agar, incubated for 6–10 weeks at 37°C, and subsequently, enumerated for CFUs using the Acolyte 3 colony counter (Lasec, CT, SA). MAB JG7 OPKA was reported as reduction of CFU in the sample wells containing JG7, compared to the control wells without MAB.

2.8. qPCR for monitoring MTB clearance from murine blood

MABs GG9 and JG7 were evaluated *in vivo* for their capacity to enhance clearance of killed MTB from blood. Female Institute of Cancer Research (ICR) mice weighing approximately 30g (Harlan Laboratories, Indianapolis, IN, USA) were given intraperitoneal injections of 300, 150 and 30μg of MABs in 0.3mL sterile PBS (10, 5 and 1 mg/kg, respectively) 24 h prior to MTB challenge. Control mice received 0.3mL of PBS. All seventy-six mice used in this study were then challenged with intravenous injection of 0.3mL of killed HN878 MTB at 10^8 CFU/mouse. Blood was obtained via retro-orbital sinus at 0.25 and 4 h post-challenge [38], and cardiac bleeds (following euthanasia) at 24 h post-challenge [33]. Approximately 0.2mL of whole blood was placed into K2 EDTA tubes

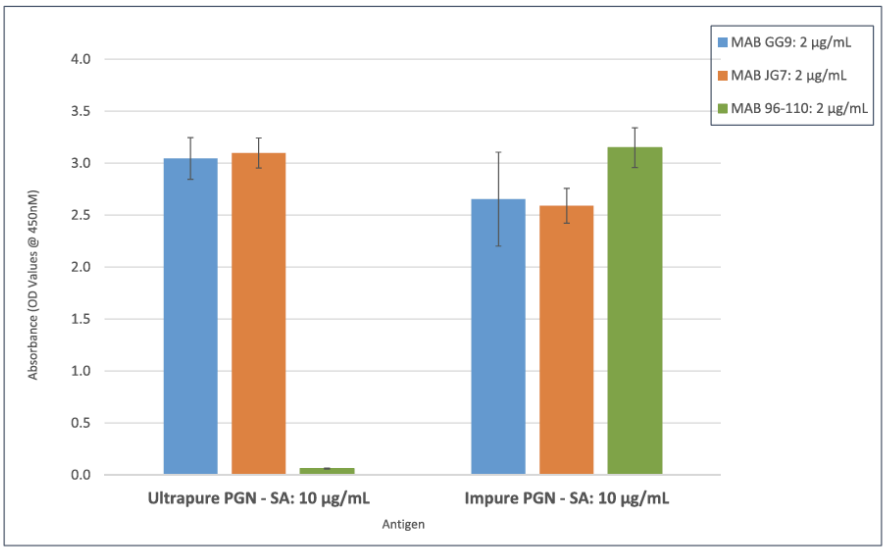

Fig. 7. PGN binding activity of MABs GG9 and JG7 was demonstrated to Ultrapure and Impure PGN, while anti-LTA MAB 96-110 only bound the Impure PGN.

(Becton Dickinson, Sparks, MD, USA), mixed gently, and then 100µL transferred into 1.0mL of PrimeStore MTM® (PS-MTM) (Longhorn Vaccines and Diagnostics, San Antonio, TX, USA). Murine blood specimens were transported overnight at ambient temperature to San Antonio, Texas for qPCR analysis. DNA extraction and multiplex qPCR were performed as described by Daum *et al* [39].

2.9. Binding of anti-MTB MABs and anti-LTA MAB to peptidoglycan

The ability of anti-MTB MABs to bind to Peptidoglycan was evaluated using Ultrapure Peptidoglycan (InvivoGen, San Diego, CA, USA) and Impure Peptidoglycan (InvivoGen, San Diego, CA, USA) both derived from *Staphylococcus aureus* to determine the target epitope for anti-MTB MABs GG9 and JG7. An anti-LTA MAB, 96-110 (Antibody and Immunoassay Consultants, Rockville, MD, USA) was used to confirm the purity of the Ultrapure Peptidoglycan from *S. aureus*. 96-well NUNC™ MaxiSorp ELISA plates (Fisher Scientific, Pittsburg, PA, USA) were coated with Ultrapure and Impure Peptidoglycan at 10µg/mL for 18–24 h at RT. Peptidoglycan-coated plates were washed with PBS-0.05% Tween 20 (PBS-T, Fisher Scientific, Pittsburg, PA, USA) using an ELx405 Automated Plate Washer (BioTek, Winooski, VT, USA). MAB samples were added and incubated for 1 h at RT. Anti-MTB antibodies were detected with HRP-Conjugated, isotype-specific Goat anti-Mouse IgG (Southern Biotech, Birmingham, AL, USA). TMB Substrate Solution (Fisher Scientific, Pittsburg, PA, USA) was added prior to being quenched with TMB STOP solution (Fisher Scientific, Pittsburg, PA, USA). The absorbance values (450nM) of each well were obtained using a SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.9.1. Statistical analysis

For *in vitro* studies, results for Live Bacteria ELISA were expressed as mean (±standard deviation) from three individual experiments. In the OPKA assays using HL60 granulocytic cells and U-937 macrophage cells, results were expressed as mean (±standard deviation) from 6 to 12 replicate sets from three individual experiments, with significance threshold set at $p < 0.05$ using the Student t-test. In the *in vivo* studies, results for each treatment group were reported as the mean (qPCR IS-6110) C_T value (±standard error) for ≥ 9 individual mice. The mean C_T values of the MAB treated groups were then compared to the mean C_T values of the placebo (PBS) treated groups at each timepoint and the differences were considered significant at $p < 0.05$ using the Student t-test. The percentage of animals that had undetectable levels of blood MTB in MAB treated versus placebo (PBS) treated groups were compared and differences analyzed using the Chi-squared test, with significance threshold set at $p < 0.05$ and 95% confidence intervals reported. Results for the Peptidoglycan ELISA were expressed as mean (±standard deviation) from three individual experiments.

3. Results

3.1. Production and Characterization of anti-MTB MABs

Subcutaneous immunization with EK-MTB elicited high ELISA antisera titers to EK-MTB. At Day 63, Mice 1323 and 1420 (each from a different boost protocol) had the strongest immune response within their respective groups with peak OD values of 4.000 and 3.134, respectively. Two hybridomas (Mouse 1323 MAB JG7 and Mouse 1420 MAB GG9) produced from spleen cell fusions, were selected for initial evaluation and demonstrated binding activity to killed MTB strains Erdman, HN878, and CDC1551 and to SMEG (Fig. 1). HN878 and CDC1551 were killed by gamma irradiation and had an intact cell surface without alcohol disruption and the MABs bound well to each strain demonstrating that the targeted epitopes were readily detected by the MABs. In addition, using susceptible MTB lab strain H37Ra and two clinical isolates the fixed MTB ELISA demonstrated good binding to both JG7 and GG9 at MAB concentrations from 10–0.156 µg/mL (Fig. 2, panel A). At 5 and 10 µg/mL, binding activity of JG7 and GG9 were each above OD 2.0 for three MDR clinical isolates (Fig. 2, panel B) and two XDR clinical isolates (Fig. 2, panel C), and their binding activity was similar to that of susceptible strains. In the Live Bacteria ELISA (Fig. 3), the binding profiles of purified MABs JG7 and GG9 were similar for SMEG at 25µg/mL. Both MABs bound to live MTB, however JG7 appeared to bind more efficiently to all MTB strains compared to GG9 and hence was selected for further OPKA studies.

3.2. OPKA of anti-MTB MABs GG9 and JG7 against live SMEG

OPKA in control wells containing complement and HL60 or U-937

cells in the absence of MAB demonstrated minimal reduction in SMEG for both HL60 or U-937 cells. Notably, OPKA was markedly increased with MAB plus complement and HL60 granulocytes, demonstrating greater than 50% OPKA above control wells with complement alone across high and low MAB concentrations ranging between 0.06–25 μg/mL (Fig. 4a). Both MABs promoted mycobacterial phagocytosis and had maximum killing of 81% for JG7 and 76% for GG9, both at 0.06μg/mL with HL60 granulocytes. These antibodies demonstrated good OPKA even at very low MAB concentrations using HL60 granulocytes and C1q (Fig. 4a). There was however a significant difference between the mean percent OPKA using JG7 compared to GG9 ($p < 0.05$) that did not change by increasing the concentration of MAB (Fig. 4a). In contrast, using U-937 macrophage cells, OPKA at antibody concentrations below 25μg/mL was statistically less robust than OPKA between 50 and 150 μg/mL ($p < 0.05$) (Fig. 4b). There was statistical significance ($p < 0.0001$) in the OPKA differences between the HL60 granulocytes and U-937 macrophages at low concentrations (< 1μg/mL) for both MABs JG7 and GG9.

3.3. OPKA of anti-MTB MABs GG9 and JG7 against live MTB

OPKA of MAB JG7 against live MTB clinical isolate STB1, using U-937 macrophages was significantly enhanced at MAB levels 2.5–25 μg/mL (Fig. 5). Compared to the control sample wells (without MAB), antibody sample wells had CFU counts that were significantly reduced ($p < 0.5$) from 315 (No MAB) to 219 (2.5μg/mL), 154 (5μg/mL), 145 (10μg/mL) and 143 (25μg/mL).

3.3.1. Dynamics of blood MTB clearance using qPCR in a mouse model

Compared to PBS-treated mice, opsonic anti-MTB MABs GG9 and JG7 enhanced clearance of MTB in mice given a high dose of killed HN878 bacilli (10^8 CFU/mouse) using MTB DNA quantification by qPCR (Fig. 6a and b). Murine blood specimens collected 0.25 h (15 min) post-challenge were qPCR positive for MTB (mean $C_T = 23.5$; $N = 76$). At 0.25 h post-challenge, MAB JG7-treated mice showed enhanced clearance of MTB from blood, compared to placebo with significant reduction in mean C_T evident at 1 mg/kg dose ($p = 0.0449$). At 4 h, blood specimens from MAB JG7-treated mice showed significant reduction in mean C_T ($p = 0.0025$, 0.0053 and 0.0232 at 10, 5 and 1 mg/kg doses, respectively) compared to placebo (Fig. 6b), while MAB GG9-treated mice were not statistically different from placebo ($p = 0.1318$, 0.1782, 0.0529) (Fig. 6a). Between 4 and 24 h, reduction in mean C_T was observed only in blood specimens from MAB GG9-treated mice (Fig. 6a). At 24 h post-challenge, mice treated with both MABs had significant reduction in mean C_T: MAB GG9, $p = 0.0013$ and 0.0021 at 10 and 1 mg/kg doses, respectively; while for MAB JG7, $p = 0.0012$ and 0.0258 at 10 and 5 mg/kg doses, respectively, compared to placebo. Notably, mean C_T reduction in MAB JG7-treated mice was statistically significant ($p < 0.05$) at 0.25 (15 min), 4 and 24 h post-challenge, while in MAB GG9-treated mice, only at 24 h post-challenge.

3.3.2. Percent of MAB-treated mice with undetectable levels of MTB in the blood, post-challenge

A significantly greater percentage of mice treated with opsonic anti-MTB MABs GG9 and JG7 given a high dose of killed HN878 bacilli (10^8 CFU/mouse) had undetectable MTB DNA in blood compared to placebo (Fig. 6c). There was no detectable MTB DNA in the blood (qPCR IS-6110 target, $C_T = 40$) at 4 and 24 h post-challenge with killed MTB in 77.8% (7/9 mice), 77.8% (7/9 mice), and 66.7% (6/9 mice) - given 1, 5 and 10 mg/kg of MAB GG9, respectively. MTB DNA was not detected in the blood at 4 and 24 h in 50% (5/10 mice), 50% (5/10 mice), and 90% (9/10 mice) - given 1, 5 and 10 mg/kg, respectively (Fig. 6c). In contrast, none of the control mice given PBS (0%, 0/19 mice) had undetectable MTB in blood at 4 and 24 h post-challenge (qPCR IS-6110, $C_T = 40$). Notably, MAB JG7, at 10 mg/kg dose, significantly enhanced clearance of MTB from blood ($p = 0.0001$, 95% CI: 48.83 to 98.21), while MAB GG9 significantly enhanced clearance of MTB from blood at 1 and 5 mg/kg doses ($p = 0.0010$, 95% CI: 33.61 to 93.69).

3.4. Anti-MTB MABs GG9 and JG7 versus anti-LTA MAB 96-110 binding activity to peptidoglycan

Purified anti-MTB MABs GG9 and JG7 each bound well to both Ultrapure and Impure PGN-SA (ODs range between 2.590 and 3.098) screened by ELISA (Fig. 7). However, the anti-LTA MAB, 96-110, only bound well to Impure PGN-SA (OD 3.149) containing cell wall LTA, and did not bind to Ultrapure PGN-SA (OD 0.061).

4. Discussion

Opsonic antibodies enhance phagocytosis and killing of encapsulated bacteria such as Group B *Streptococcus in vitro*, and enhance protection *in vivo* [19, 20, 21]. Monoclonal antibodies that bind to MTB capsule components, e.g. arabinomannan (AM), promote mycobacterial opsonization, phagocytosis, induced intracellular mycobacterial growth reduction, P-L fusion [24], and prolonged survival in mice [40, 41]. Using THP-1 macrophage cells, Chen *et al.*, showed that enhancement of BCG phagocytosis and reduction in growth rate correlated with AM epitope specific IgG [42]. In addition, studies by Lu and colleagues suggest that antibodies could direct inflammasome activation in macrophages that may contribute to bacterial control [43]. In this study, we demonstrate that IgG MABs directed against MTB promote OPKA *in vitro* and enhance blood clearance of killed MTB *in vivo*. While qPCR can rapidly monitor the removal of MTB DNA from the blood, it does not indicate if the MTB have been killed by the phagocytic cells, but MTB OPKA was shown to be enhanced by MAB JG7.

Immunizing mice with EK-MTB may have altered the capsule exposing deeper cell wall epitopes. Previous studies have shown that cell wall lipoteichoic acid (LTA) antibodies promote phagocytosis and killing of staphylococci and enhanced blood clearance *in vivo* [44, 45]. Data from the studies presented here using highly purified peptidoglycan strongly suggest that the target of MABs GG9 and JG7 is an epitope on peptidoglycan. These anti-peptidoglycan MABs bind to antibiotic sensitive and resistant live MTB and promote opsonophagocytic killing of MTB by U-937 macrophage cells. Kumar *et al.*, observed that not all antibodies to MTB in serum of individuals with TB took part in facilitating opsonization of MTB [32]. It is not clear whether anti-peptidoglycan antibodies are induced in individuals with TB infections.

Both granulocytes and macrophages are important for clearance of microbes from the blood and body tissues. When compared to PBS, MABs GG9 and JG7 enhanced MTB clearance in ICR mice given an intravenous injection of killed HN878. The clearance of killed MTB from murine blood was monitored by qPCR at a distant laboratory. This method of collecting blood, safely transporting specimens at ambient temperature to a distant lab, and analyzing samples using qPCR [39] may be useful for detecting MTB sepsis, determining the level of bacteremia and monitoring clearance of MTB in patients with MTB sepsis.

Immunotherapy may be useful to improve the treatment of bacteremic MTB infections. Antibodies that bind to MDR and XDR MTB, promote bactericidal phagocytic activity and enhance clearance of MTB bacteremia may provide adjunctive therapy for MTB sepsis, and augment treatment with antibiotics for patients with MDR and XDR TB. A larger comprehensive evaluation of these MABs using live MTB and *in vivo* infection models will be critical to determine if these MABs might be effective in treating patients with MTB sepsis or MDR/XDR TB.

Declarations

Author contribution statement

Clara J. Sei, Bong-Akee Shey, Richard F. Schuman, Nimisha Rikhi, Luke T. Daum, P Bernard Fourie, Gerald W. Fischer: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.

Clara J. Sei, Bong-Akee Shey, Richard F. Schuman, Nimisha Rikhi,

Luke T. Daum, Kevin Muema, John D. Rodriguez: Performed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

[1] World Health Organization, Global Tuberculosis Report, 2018. Available at: http://www.who.int/tb/publications/global_report/en/. (Accessed 18 September 2018).
[2] World Health Organization, Tuberculosis Fact Sheet N°104, 2018. Available at: http://www.who.int/mediacentre/factsheets/fs104/en/. (Accessed 18 September 2018).
[3] E.L. Corbett, B. Marston, G.J. Churchyard, K.M. DeCock, Tuberculosis in sub-Saharan Africa: opportunities, challenges and change in the year of antiretroviral treatment, Lancet 367 (2006) 926–937.
[4] N.A. Martinson, A. Karstaedt, W.D.F. Venter, T. Omar, P. King, et al., Causes of death in hospitalized adults with a premortem diagnosis of tuberculosis: an autopsy study, AIDS 21 (2007) 2043–2050.
[5] J.A. Cox, R. Lukande, A.M. Nelon, H. Mayanja-Kizza, R. Clebunders, et al., An autopsy study describing causes of death and comparing clinicopathological findings among hospitalized patients in Kampala, Uganda, PLoS One 7 (3) (2012) e33685.
[6] C.F. Gilks, R.J. Brindle, L.S. Otieno, P.M. Simani, R.S. Newnham, et al., Life-threatening bacteraemia in HIV-1 seropositive adults admitted to hospital in Nairobi, Kenya, Lancet 336 (1990) 545–549.
[7] L.K. Archibald, M.O. den Dulk, J.K. Pallangyo, L.B. Reller, Fatal *Mycobacterium tuberculosis* bloodstream infections in febrile hospitalized adults in Dar es Salaam, Tanzania, Clin. Infect. Dis. 26 (1998) 290–296.
[8] L.C. McDonald, L.K. Archibald, S. Rheanpumikankit, S. Tansuphaswadikul, B. Eampokalap, et al., Unrecognized *Mycobacterium tuberculosis* bacteraemia among hospital inpatients in less developed countries, Lancet 354 (1999) 1159–1163.
[9] D.K. Lewis, R.P. Peters, M.J. Schijffelen, G.R. Joaki, A.L. Walsh, et al., Clinical indicators of mycobacteraemia in adults admitted to hospital in Blantyre, Malawi, Int. J. Tuberc. Lung Dis. 6 (12) (2002) 1067–1074.
[10] J.A. Crump, H.O. Ramadhani, A.B. Morrissey, W. Saganda, M.S. Mwako, et al., Bacteremic disseminated tuberculosis in sub-Saharan Africa: a prospective cohort study, Clin. Infect. Dis. 55 (2) (2012) 242–250.
[11] P.J. Munseri, E.A. Talbot, M. Bakari, M. Matee, J.P. Teixeira, et al., The bacteraemia of disseminated tuberculosis among HIV-infected patients with prolonged fever in Tanzania, Scand. J. Infect. Dis. 43 (9) (2011) 696–701.
[12] S.S. Ahuja, S.K. Ahuja, K.R. Phelps, W. Thelmo, A.R. Hill, Hemodynamic confirmation of septic shock in disseminated tuberculosis, Crit. Care Med. 20 (6) (1992) 901–903.
[13] R.A. Ferrand, J. Herman, A. Elgalib, J. Cartledge, R.F. Miller, Septic shock and multi-organ failure in HIV infection-'sepsis tuberculosa gravissima', Int. J. STD AIDS 17 (2006) 562–564.
[14] S.T. Jacob, C.C. More, P. Banura, R. Pinkerton, D. Meya, et al., Severe sepsis in two Ugandan hospitals: a prospective observational study of management and outcomes in a predominantly HIV-1 infected population, PLoS One 4 (11) (2009) e7782.
[15] S.T. Jacob, P.B. Pavlinac, L. Nakiyingi, P. Banura, et al., *Mycobacterium tuberculosis* bacteremia in a cohort of HIV-infected patients hospitalized with severe sepsis in Uganda-high frequency, low clinical sand derivation of a clinical prediction score, PLoS One 8 (8) (2013) e70305.
[16] E.M. Streicher, B. Müller, V. Chihota, C. Mlambo, M. Tait, et al., Emergence and treatment of multidrug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis in South Africa, Infect. Genet. Evol. 12 (2012) 686–694.
[17] W.H. Park, J.G.M. Bullowa, M.B. Rosenbluth, The treatment of lobar pneumonia with refined specific antibacterial serum, J. Am. Med. Assoc. 91 (20) (1928) 1503–1508.
[18] M. Singer, S. Nambiar, T. Valappil, K. Higgins, S. Gitterman, Historical and regulatory perspectives on the treatment effect of antibacterial drugs for community-acquired pneumonia, Clin. Infect. Dis. 47 (3) (2008) S216–S224.
[19] G.W. Fischer, Immunoglobulin therapy of neonatal group B streptococcal infection: an overview, Pediatr. Infect. Dis. J. 7 (1988) S13.
[20] G.W. Fischer, Therapeutic uses of intravenous gammaglobulin for pediatric infections, Pediatr. Clin. N. Am. 35 (1988).
[21] Z. An, Antibody therapeutics – a mini review, Trends Bio/Pharma. Indus. 2 (2008) 24–29.
[22] K.R. Steingart, N. Dendukuri, M. Henry, I. Schiller, P. Nahid, et al., Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a meta-analysis, J. Clin. Vaccine Immunol. 16 (2009) 260–276.
[23] M. Daffe, G. Etienne, The capsule of *Mycobacterium tuberculosis* and its implications for patho-genicity, Tuber. Lung Dis. 79 (3) (1999) 153–169.
[24] T. Chen, C. Blanc, A.Z. Eder, R. Prados-Rosales, A.C.O. Souza, et al., Association of human antibodies to arabinomannan with enhanced mycobacterial opsonophagocytosis and intracellular growth reduction, J. Infect. Dis. 214 (2) (2016) 300–310.
[25] R.W. Stokes, R. Norris-Jones, D.E. Brooks, T.J. Beveridge, D. Doxsee, et al., The glycan-rich outer layer of the cell wall of *Mycobacterium tuberculosis* acts as an antiphagocytic capsule limiting the association of the bacterium with macrophages, Infect. Immun. 72 (10) (2004) 5676–5686.
[26] B. Dey, W.R. Bishai, Crosstalk between *Mycobacterium tuberculosis* and the host cell, Semin. Immunol. 26 (6) (2014) 486–496.
[27] S. Ahmad, Pathogenesis, immunology, and diagnosis of latent *Mycobacterium tuberculosis* infection, J. Clin. Develop. Immunol. 2011 (17) (2011).
[28] J.M. Achkar, A. Casadevall, Antibody-mediated immunity against tuberculosis: implications for vaccine development, Cell Host Microbe 13 (3) (2013) 250–262.
[29] A. Lemassu, M. Daffé, Structural features of the exocellular polysaccharides of *Mycobacterium tuberculosis*, Biochem. J. 297 (Pt 2) (1994) 351–357.
[30] J.M. Achkar, J. Chan, A. Casadevall, B cells and antibodies in the defense against *Mycobacterium tuberculosis* infection, Immunol. Rev. 264 (1) (2015) 167–181.
[31] M. Podinovskaia, W. Lee, S. Caldwell, D.G. Russell, Infection of macrophages with *Mycobacterium tuberculosis* induces global modifications to phagosomal function, Cell Microbiol. 15 (6) (2013) 843–859.
[32] S.K. Kumar, P. Singh, S. Sinha, Naturally produced opsonizing antibodies restrict the survival of *Mycobacterium tuberculosis* in human macrophages by augmenting phagosome maturation, Open Biol. 5 (12) (2015) 150171.
[33] AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.
[34] A.H. Bartal, Y. Hirshaut, Methods of Hybridoma formation. Issue 2, 7, Humana Press, Clifton New Jersey, 1987, p. 154.
[35] M. Shulman, C.D. Wilde, G. Kohler, A better cell line for making hybridomas secreting specific antibodies, Nature 276 (1978) 269–270.
[36] G. Wiederschain, Affinity Chromatography. Methods and protocols, Biochem. Moscow - Biochem. Engl Tr 73 (2008) 1169.
[37] R.A. Fleck, S. Romero-Steiner, M.H. Nahm, Use of HL-60 cell line to measure opsonic capacity of pneumococcal antibodies, Clin. Diagn. Lab. Immunol. (2005) 19–27.
[38] S. Parasuraman, R. Raveendran, R. Kesavan, Blood sample collection in small laboratory animals, J. Pharmacol. Pharmacother. 1 (2) (2010) 87–93.
[39] L.T. Daum, R.F. Schuman, C.J. Sei, N. Rikhi, A. Mesadieu, et al., Rapid qPCR detection of *Mycobacterium tuberculosis* in blood and organ tissues using a collection-to-detection system, Enliven: Micro. Microbiol. Tech. 4 (1) (2017) 002.
[40] B. Hamasur, M. Haile, A. Pawlowski, U. Schröder, G. Källenius, et al., A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab')2 fragment prolong survival of mice infected with *Mycobacterium tuberculosis*, Clin. Exp. Immunol. 138 (1) (2004) 30–38.
[41] R. Teitelbaum, M. Cammer, M.L. Maitland, N.E. Freitag, J. Condeelis, et al., Mycobacterial infection of macrophages results in membrane-permeable phagosomes, Proc. Natl. Acad. Sci. U. S. A 96 (26) (1999) 15190–15195.
[42] T. Chen, C. Blanc, A.Z. Eder, R. Prados-Rosales, A.C.O. Souza, et al., Association of human antibodies to arabinomannan with enhanced mycobacterial opsonophagocytosis and intracellular growth reduction, J. Infect. Dis. 214 (2) (2016) 300–310.
[43] L.L. Lu, A.W. Chung, T. Rosebrock, M. Ghebremichael, W.H. Yu, et al., A functional role for antibodies in tuberculosis, Cell 167 (2) (2016) 433–443, e14.
[44] L.E. Weisman, H.M. Thackray, J.A. Garcia-Prats, M. Nesin, J.H. Schneider, et al., Phase 1/2 double-blind placebo controlled, dose escalation, safety, and pharmacokinetic study of pagibaximab (BSYX-A110), an anti-staphylococcal monoclonal antibody for the prevention of staphylococcal bloodstream infections in very low birth weight neonates, Antimicrob. Agents Chemother. 53 (7) (2009) 2879–2886.
[45] L.E. Weisman, H.M. Thackray, R.H. Steinhorn, W.E. Walsh, H.A. Lassiter, et al., A randomized study of a monoclonal antibody (pagibaximab) to prevent staphylococcal sepsis, Pediatrics 128 (2) (2011) 271–279.

Chapter 4

4 Demonstrating novel IgG1 monoclonal antibody-enhanced phagocytosis of *Mycobacterium tuberculosis* by macrophages and granulocytes: An *in vitro* study

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4.1 Overview

Summary: In this Chapter, results are presented for *in vitro* opsonophagocytic (OP) assays conducted on two drug susceptible *M. tuberculosis* strains (laboratory strain H37Ra and a clinical strain) using the human macrophage and granulocyte cell lines U-937 and HL-60 respectively. The complete experimental procedures and supplementary data for this chapter are provided in Appendices A, B, and C.

Contribution to the thesis and novelty: This chapter expands on earlier observations of *in vitro* monoclonal antibody (mAb)-enhanced phagocytosis or opsonophagocytosis of *Mycobacterium tuberculosis*, using the human U-937 macrophage cell line. New findings are presented on the effect of the novel IgG1 mAb (JG7) on opsonophagocytosis.

Contributions of Candidate: All work involving the *in vitro* OP assays, from bacterial sub-culture to cell culture techniques, and colony enumeration, has been performed by the candidate at UP. The candidate conducted preliminary analysis and led the interpretation of the results in line with the stated objectives of the overall thesis title.

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4.2 Abstract

Host-directed therapies (HDTs) are increasingly being explored as an approach for enhancing treatment outcomes in patients with tuberculosis (TB). Monoclonal antibodies (mAbs) of the immunoglobulin gamma (IgG) class have been shown *in vitro*, to possess immunotherapeutic properties against *Mycobacterium tuberculosis* (*M. tuberculosis*), with reactivity to epitopes present on both live and killed *M. tuberculosis* cells. The development of an HDT based on mAbs requires, primarily, the demonstration of high mycobacterial cell-wall binding activity by the mAb, and secondly, enhancement of phagocytic activity leading to *M. tuberculosis* elimination.

The ability of the selected novel mouse IgG1 mAb JG7 III D3 I F9 (JG7) to bind to both susceptible and drug-resistant *M. tuberculosis* strains and the functional capacity of JG7 to enhance the phagocytic elimination of susceptible *M. tuberculosis* by the human macrophage (U-937) cell line formed the basis for expanded research into opsonophagocytic killing activity. In this paper, further evidence that different concentrations of JG7 enhance the phagocytosis and killing of different dilutions of susceptible *M. tuberculosis* (both H37Ra ATCC 25177 and a clinical isolate [cMtb]) in both human macrophage (U-937) and granulocyte (HL-60) cell lines, is provided. Eight JG7 concentrations and two bacterial dilutions were used in at least three experiments per cell-line for each of the two susceptible *M. tuberculosis* strains (H37Ra and cMtb) investigated. The percentage colony-forming unit (CFU) reduction for each sample containing JG7, relative to the control without JG7, formed the basis of observations on effects.

The performance of each JG7 concentration, demonstrated by opsonophagocytic killing activity (OPKA) calculated from the data obtained for the two bacterial dilutions 1:100 and 1:1000, was least effective at the lower JG7 concentrations (0.25 µg/mL and 0.5 µg/mL), and more effective at the middle range of concentrations (1 µg/mL, 2.5 µg/mL, and 10 µg/mL) as well as the higher concentrations (50 µg/mL and 100 µg/mL). Overall, the 10 µg/mL concentration performed well with the U-937 cell line while the 1 µg/mL concentration performed well with the HL-60 cell line. The research methodology allows for the assessment of the magnitude of *M. tuberculosis* clearance, thereby offering an approach for determining the efficacy of potential candidates for HDTs against TB.

Keywords: *M. tuberculosis*, monoclonal antibody (mAb), immunoglobulin gamma 1 (IgG1), *in vitro*, host-directed therapy (HDT), opsonophagocytic killing activity (OPKA)

4.3 Introduction

The current interventions against pulmonary tuberculosis (TB) such as chemotherapy and vaccination have not been enough to combat the disease. This is evident by the increase in treatment failure, relapse, and re-infection rates [Colangeli *et al*., 2018, Romanowski *et al*., 2018]. Finding alternative therapeutic approaches for eradicating TB has become critical. In addition, treatment options that could prevent latent *M. tuberculosis* infection from progressing to active disease would significantly impact TB control. Host-directed therapy (HDT) or immunotherapy is potentially one such intervention, holding promise for shortening the length of treatment for both susceptible and drug resistant TB, reducing the risk for TB recurrence/relapse after chemotherapy, and even enhancing the treatment success for resistant TB [Roy *et al*., 2007, Zumla *et al*., 2015]. In the presence of increasing *M. tuberculosis* resistance, HDT is becoming an important alternative and a promising adjunctive therapy for enhancing treatment outcomes [Balu *et al*., 2011, Zumla *et al*., 2013, Achkar and Casadevall, 2013, Zumla *et al.*, 2015].

Monoclonal antibodies (mAbs) of the immunoglobulin gamma (IgG) class have been shown to possess immunotherapeutic properties against *M. tuberculosis in vitro,* with reactivity to epitopes present on both live and killed *M. tuberculosis* cells [Balu *et al*., 2011, Welch *et al*., 2012]. Measuring mAb-enhanced *M. tuberculosis* elimination as a therapeutic option would require methods, to firstly, demonstrate high mycobacterial cell-wall binding activity and secondly, demonstrate enhanced phagocytic activity in the presence of the mAb.

A novel mouse IgG1 mAb, JG7 III D3 I F9 (JG7), selected from a broad panel of novel mAbs (unpublished data) was previously evaluated, and it was demonstrated that, this mAb had the ability to bind to both susceptible and resistant *M. tuberculosis* strains at different concentrations [Sei *et al.*, 2019]. The specific antigenic epitopes or binding receptors were not identified, but there is suspicion that JG7 binds to an epitope involving *M. tuberculosis* peptidoglycan [Sei *et al*., 2019]. In addition, Chen *et al*., (2016) have shown that the arabinomannan component of the *M. tuberculosis* cell wall is a second possible target, by demonstrating that arabinomannan is inhibited by an increase in IgG antibody-enhanced phagocytosis of *M. tuberculosis*.

This study aimed to determine the suitability of novel mouse IgG1 mAb JG7, to enhance the phagocytic and antimicrobial activities of human macrophage and granulocyte cell lines against different susceptible *M. tuberculosis* strains. The hybridoma mouse mAb JG7 was produced from

mouse spleen cell fusions after subcutaneous immunization of these mice with ethanol-killed *M. tuberculosis.* The letters and numbers on the mAb refer to the mouse hybridoma clone and allow tracing of the history and background of the clone for mAb development.

Human cancer cell lines were used because these cell lines have been well characterized, and due to their durability since they can remain viable for longer periods of time, as well as to limit any experimental variability which may occur as a result of isolating different human cells from different individuals. Reproducibility and consistency had also been demonstrated for these two human cell lines, with previous work conducted on *Mycobacterium smegmatis* (Chapter 3).

In order to determine the suitability of mAb JG7 for opsonophagocytosis, four experimental objectives were investigated, based on the following hypotheses:

1. mAb JG7 enhances the phagocytosis of *M. tuberculosis* H37Ra (ATCC 25177) in U-937 human macrophage cells.
2. mAb JG7 enhances the phagocytosis of drug susceptible *M. tuberculosis* (clinical isolate [cMtb]), in U-937 human macrophage cells.
3. mAb JG7 enhances the phagocytosis of *M. tuberculosis* H37Ra (ATCC 25177) in HL-60 human granulocyte cells.
4. mAb JG7 enhances the phagocytosis of drug susceptible *M. tuberculosis* (cMtb), in HL-60 human granulocyte cells.

4.4 Materials and methods

4.4.1 Strain description

Pure cultures of the laboratory strain *M. tuberculosis* H37Ra (ATCC 25177) supplied by Thermo Fisher Scientific, Massachusetts, USA, and a susceptible *M. tuberculosis* clinical isolate (NHLS/TAD, Pretoria, South Africa) were grown in the BACTEC 960 system containing mycobacterial growth incubator tube (MGIT) 7H9 broth (Becton Dickinson, New Jersey, USA), at 37ºC until either mid-logarithmic phase ($1x10^7$ CFU/mL - $1x10^8$ CFU/mL) or stationary phase ($1x10^8$ CFU/mL or $1x10^9$ CFU/mL).

4.4.2 Bacterial dilution

Two bacterial serial dilutions were used in each opsonophagocytic (OP) assay run (1:100 and 1:1000 for mid-logarithmic phase bacteria; or 1:1000 and 1:10 000 for stationary phase bacteria), in order to assess if different bacterial concentrations would have any effect on phagocytosis, within each human cell line. The detailed experimental procedures for U-937 and HL-60 cell passaging and differentiation, are presented below and in **Appendix B** of the supplementary material.

4.4.3 Passaging and differentiation of U-937 macrophage cells for phagocytosis

The Human Histiocytic Lymphoma cell line U-937 (ATCC, Manassas, VA) was passaged every 3 to 4 days using complete growth medium (CGM), at 37ºC in a 5% carbon dioxide (CO_2) incubator (Lasec, Cape Town, South Africa). U-937 cells were passaged a maximum of 12 times before starting over with a new batch of cells to ensure optimal performance of the cells. Cell viability was determined by counts performed under a light microscope (Zeiss, Cape Town, South Africa) at x40 objective and the cells were only differentiated if viability was ≥ 90%. Furthermore, the cells were only differentiated for the OP assays from the 4th cell passage in order to ensure cell maturity. Differentiation of U-937 cells was performed in CGM containing Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich GmbH, Munich, Germany) at 2x10^6 cells/mL for 3 to 4 days in sterile 96-well round-bottom polystyrene plates (Lasec, Cape Town, South Africa). Differentiation medium was taken out on the second day of differentiation and the cells re-suspended in CGM in order to avoid induced mortality of cells by PMA.

4.4.4 Passaging and differentiation of HL-60 granulocyte cells for phagocytosis

The Human Leukaemia Promyelocytic cell line HL-60 (Sigma-Aldrich, St. Louis, MO; ATCC, Manassas, VA) was passaged in a similar way as the U-937 cells (see 4.4.3 above). HL-60 cells were passaged a maximum of 14 times before starting over with a new batch of cells in order to ensure optimal performance of the cells. The cells were differentiated for the OP assays from the 3rd cell passage in order to ensure cell maturity. Differentiation of HL-60s was performed in CGM containing 12% foetal bovine serum (FBS) (Lasec, Cape Town, South Africa) and 1.25% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich GmbH, Munich, Germany) at 1x10^5 cells/mL for 5 - 6 days in T-175 flasks (Thermo Fisher Scientific, Massachusetts, USA).

4.4.5 *In vitro* opsonophagocytic assay

mAb JG7 concentration: JG7 (Longhorn Vaccines and Diagnostics, San Antonio, TX) was diluted in cold OP assay medium at eight concentrations ranging from 0.25 µg/mL to 100 µg/mL in order to assess the effect of mAb concentration on the phagocytosis of *M. tuberculosis* within each human cell line.

Plate set-up: A volume of 40 µL of each concentration of JG7 followed by 10 µL of mid-logarithmic or stationary phase *M. tuberculosis* (H37Ra or cMtb) at one of two dilutions (1:100 and 1:1000 for bacteria at 10^8 CFU/mL, or 1:1000 and 1:10 000 for bacteria at 10^9 CFU/mL), was added directly into the wells of a 96-well plate (Thermo Fisher Scientific, Massachusetts) containing 50 µL of the cells (40 µL of HL-60 cells plus 10 µL of activating complement component C1q (Biocom Diagnostics, South Africa).

Each of the two bacterial dilutions was then run in triplicate (3 wells) across eight JG7 concentrations while controls without JG7 were run in six wells (three wells for each of the bacterial dilution groups), as shown in **Table 4.1**.

Table 4.1: Plate set-up for the *in vitro* opsonophagocytic assay

96-well plate		**Cell line**											
		Bacterial dilution 1			Bacterial dilution 2			Bacterial dilution 1			Bacterial dilution 2		
mAb JG7 concentration (µg/mL)		***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***
	A	T8	T8	T8	T8	T8	T8	T4	T4	T4	T4	T4	T4
	B	T7	T7	T7	T7	T7	T7	T3	T3	T3	T3	T3	T3
	C	T6	T6	T6	T6	T6	T6	T2	T2	T2	T2	T2	T2
	D	T5	T5	T5	T5	T5	T5	T1	T1	T1	T1	T1	T1
	E	T0	T0	T0	T0	T0	T0	T0	T0	T0	T0	T0	T0
	F	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	-	-	-	-	-	-	-	-	-	-

Cell line: U-937 (human macrophage) or HL-60 (human granulocyte)

Bacterial dilution 1: H37Ra or cMtb isolate grown to 10^8 CFU/mL, harvested mid-log and diluted 1:100 or grown to 10^9 CFU/mL, harvested stationary phase and diluted 1:1000

Bacterial dilution 2: H37Ra or cMtb isolate grown to 10^8 CFU/mL, harvested mid-log and diluted 1:1000 or grown to 10^9 CFU/mL, harvested stationary phase and diluted 1:10 000 (indicated as such in the analysis)

mAb JG7 concentration: T (treatment); T8=100 µg/mL; T7=50 µg/mL; T6=25 µg/mL; T5=10 µg/mL; T4=2.5 µg/mL; T3=1.0 µg/mL; T2=0.5 µg/mL; T1=0.25 µg/mL; T0=0 µg/mL (control).

Plate incubation: The plates were sealed and incubated at 37ºC for 4 hours. After incubation, 90 µL of the mixture was discarded from each well and 190 µL of ice cold 0.1% BSA (30% BSA [Sigma-Aldrich GmbH, Munich, Germany] in sterile grade water) was added to each well in order to lyse the cells. The plates were incubated on ice for 30 minutes. Thereafter, 100 µL of each reaction mixture was taken from each well and plated on Middlebrook 7H10 or 7H11 agar (Becton Dickinson, New Jersey, USA), incubated for 6 - 10 weeks at 37ºC, and subsequently enumerated for CFUs using the Acolyte 3 colony counter (Lasec, Cape Town, South Africa).

Factors assessed for opsonophagocytic killing activity in the human cell lines: Strain type, mAb concentration, and bacterial dilution were investigated as primary determinants of the phagocytic action of both U-937 macrophage and HL-60 granulocyte cells.

Statistics: Opsonophagocytic killing activity (OPKA) was defined as a decrease in the average number of bacterial CFUs in sample wells, compared to the average number of CFUs in control wells containing no mAb. Analytical statistics made use of margins and linear prediction models to compare the increase or decrease in OPKA for each of eight treatments (T1-T8, represented by different concentrations of JG7) over controls (T0). An increase in OPKA was considered statistically significant when the p-value was less than 0.05.

4.5 Results

4.5.1 Data obtained from opsonophagocytic assays with U-937 macrophage cell line

OP assay data for H37Ra and cMtb with U-937 macrophage cells is provided in **Appendix C**, supplementary **Sheets 1-2**. Descriptive data for both H37Ra and cMtb are presented per JG7 concentration and per bacterial dilution in **Table 4.2** below, which is a representative data set showing the average bacterial CFUs of the total number of observations in sample and control wells per bacterial dilution for each JG7 concentration used with the U-937 cell line.

Analytical statistics made use of margins and linear prediction models (**Table 4.3**). Eight mAb concentrations were explored, labelled as treatments 1 to 8 (T1-T8), ranging from the lowest concentration (0.25 µg/mL) to the highest concentration (100 µg/mL). Treatment 0 represents the experimental control without mAb (baseline value).

The increase or decrease in phagocytosis for each of these eight treatments compared with the baseline or treatment 0, is shown at 95% confidence intervals. An increase in OPKA was considered statistically significant when the p-value was less than 0.05.

Table 4.2: Effect of JG7 at different concentrations on bacterial CFUs by strain type and bacterial dilution, in the U-937 macrophage cell line

Strain: H37Ra									
JG7 concentration* (µg/mL)	Bacterial dilution**								
	1:100			1:1000			1:10 000		
	N	CFU		N	CFU		N	CFU	
		Mean	SD		Mean	SD		Mean	SD
0	12	155.0	120.1	24	164.3	226.8	12	141.9	85.2
0.25	6	126.8	66.1	12	137.8	190.4	6	82.2	20.2
0,5	6	117.2	26.8	12	161.9	196.5	6	103.2	30.4
1	6	183.0	61.7	12	176.7	226.0	6	55.0	33.1
2.5	6	143.3	97.6	12	100.7	114.8	6	73.3	50.4
5	3	108.0	111.7	3	10.7	18.5	-	-	-
10	6	92.0	29.5	12	106.8	111.7	6	93.7	45.7
15	3	197.3	21.4	3	0.0	0.0	-	-	-
25	6	68.8	27.5	12	142.6	196.9	6	85.3	42.8
50	3	135.7	28.7	9	201.9	243.7	6	131.0	123.0
100	3	108.3	52.0	9	168.9	184.2	6	94.8	67.3

Strain: cMtb									
JG7 concentration* (µg/mL)	Bacterial dilution**								
	1:100			1:1000			1:10 000		
	N	CFU		N	CFU		N	CFU	
		Mean	SD		Mean	SD		Mean	SD
0	18	516.1	291.9	23	462.9	196.1	6	579.0	108.2
0.25	9	546.9	375.3	12	395.3	239.9	3	552.7	54.0
0.5	9	421.0	320.4	12	401.8	274.3	3	433.0	92.7
1	9	455.1	325.2	12	400.2	216.2	3	444.3	74.3
2.5	9	423.7	308.2	12	368.3	216.6	3	506.0	91.2
5	3	282.0	22.5	3	340.0	136.7	-	-	-
10	9	361.6	241.6	12	387.6	184.3	3	446.7	74.8
15	3	227.3	56.7	3	351.0	60.2	-	-	-
25	9	392.3	355.5	12	337.2	146.4	3	471.3	12.4
50	6	477.0	348.6	9	361.8	189.1	3	450.0	139.5
100	6	564.8	450.1	9	483.2	311.7	3	416.7	74.1

H37Ra (susceptible laboratory strain, ATCC 25177), cMtb (susceptible patient strain, clinical *M. tuberculosis*), N (number of observations), CFU (colony forming units), SD (standard deviation)

* 5 µg/mL and 15 µg/mL concentrations represent fewer observations because they were used in only one experiment, with the exclusion of 50 µg/mL and 100 µg/mL. These out-of-range observations were excluded from the overall data analysis

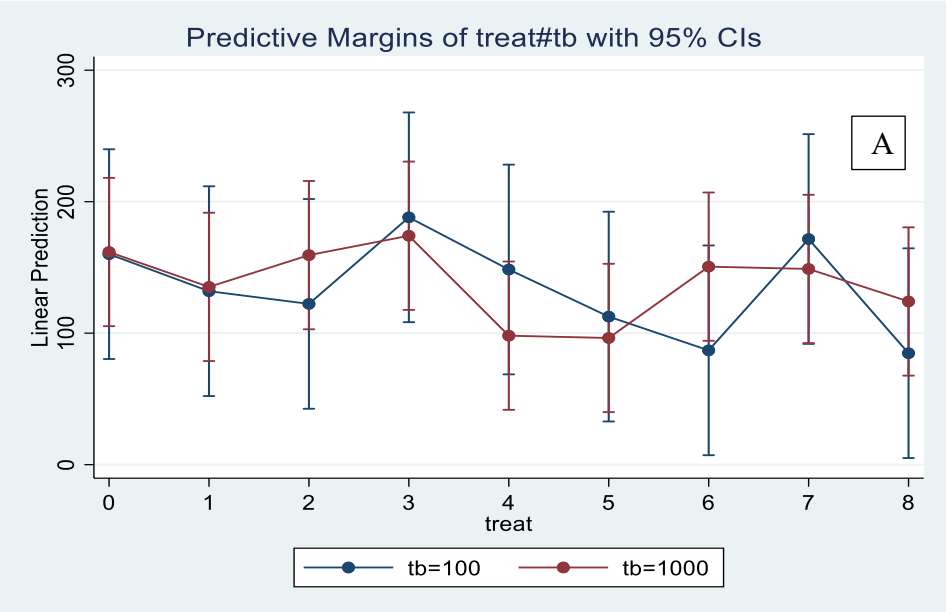
** Starting concentrations for dilution 1:100 were $1x10^8$ CFU/mL, $1x10^8$ CFU/mL or $1x10^9$ CFU/mL for dilution 1:1000, and $1x10^9$ CFU/mL for 1:10 000

Table 4.3: Comparing H37Ra dilutions 1:100 and 1:1000 (p-value, 0.775) by JG7 concentration (T0-T8) for effect on bacterial CFUs in U-937 macrophages

Treatment	Margin	t-value	95% CI	dy/dx	p-value
0	161.19	7.11	115.15 - 207.24		
1	134.11	5.91	88.06 - 180.16	-27.08	0.404
2	147.00	6.48	100.95 - 193.05	-14.19	0.661
3	178.78	7.88	132.73 - 224.83	17.58	0.587
4	114.89	5.07	68.84 - 160.94	-46.31	0.158
5	101.78	4.49	55.73 - 147.83	-59.42	0.072
6	129.39	5.70	83.34 - 175.44	-31.81	0.328
7	156.44	6.90	110.40 - 202.50	-4.75	0.883
8	111.00	4.89	64.95 - 157.05	-50.19	0.127

Treatment=mAb JG7 concentration (µg/mL): T8=100; T7=50; T6=25; T5=10; T4=2.5; T3=1.0; T2=0.5; T1=0.25; T0=0 (control); CI=confidence interval

Figure 4.1 is a margins plot drawn from **Table 4.3**, showing the effects that treatments 1 to 8 (JG7 concentrations 0.25 µg/mL to 100 µg/mL respectively) have on bacterial CFUs relative to treatment 0 (control) at H37Ra dilutions 1:100 and 1:1000. This analysis excludes consideration of data for the 1:10 000 dilution, given the fact that comparatively, the total number of observations were too sparse to allow for statistical conclusions to be adequately drawn. **Figure 4.1** (A) represents effects of T1-T8 when data obtained at H37Ra dilutions 1:100 and 1:1000 are considered separately, while **Figure 4.1** (B) represents effects of T1-T8 when data obtained at H37Ra dilutions 1:100 and 1:1000 are combined.



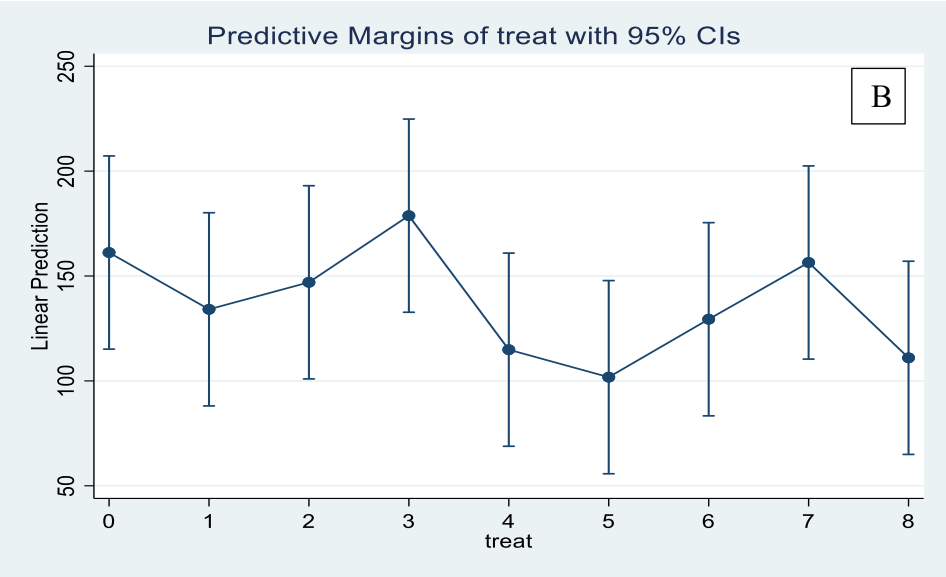


Figure 4.1: Predictive margins of change in H37Ra CFUs by JG7 concentration for macrophage cell line U-937 and bacterial dilutions 1:100 and 1:1000 (A=Individually; B=Combined)

(treat 0 = baseline/control with no JG7, treat 1 = 0.25 µg/mL, treat 2 = 0.5 µg/mL, treat 3 = 1 µg/mL, treat 4 = 2.5 µg/mL, treat 5 = 10 µg/mL, treat 6 = 25 µg/mL, treat 7 = 50 µg/mL, treat 8 = 100 µg/mL)

According to **Figure 4.1** (B), relative to baseline CFU values for H37Ra without mAb exposure, OPKA was only marginally significant ($0.05 \leq p \leq 1$, at a 95% confidence interval) at all eight JG7 concentrations. However, there is a noticeable trend in bacterial CFU increase after a marked decrease (from T1 - T3 and from T5 - T7). Similar to the analysis above, **Figure 4.2** is a margins plot drawn from **Table 4.4**, showing the effects that T1 to T8 (i.e. JG7 concentrations 0.25 µg/mL to 100 µg/mL respectively) have on bacterial CFUs relative to baseline (T0, control) at cMtb dilutions 1:100 and 1:1000. This analysis excludes consideration of data for the 1:10 000 dilution.

Table 4.4: Comparing cMtb dilutions 1:100 and 1:1000 (p-value, 0.048) by JG7 concentration (T0-T8) for effect on bacterial CFUs in U-937 macrophages

Treatment*	Margin	t-value	95% CI	dy/dx	p-value
0	470.29	17.46	415.99 - 524.58		
1	455.52	16.91	401.23 - 509.82	-14.76	0.700
2	405.24	15.04	350.95 - 459.54	-65.05	0.095
3	406.67	15.10	352.37 - 460.96	-63.62	0.102
4	389.29	14.45	334.99 - 443.58	-81.00	0.039
5	369.10	13.70	314.80 - 423.39	-101.19	0.011
6	376.81	13.99	322.52 - 431.10	-93.48	0.018
7	365.62	13.57	311.33 - 419.91	-104.67	0.009
8	441.86	16.40	387.56 - 496.15	-28.43	0.460

*Treatment=mAb JG7 concentration (µg/mL): T8=100; T7=50; T6=25; T5=10; T4=2.5; T3=1.0; T2=0.5; T1=0.25; T0=0 (control).; CI=confidence interval

According to **Figure 4.2**, relative to baseline CFU values for cMtb without mAb exposure, a steady decrease in bacterial CFUs could be observed from T1 (0.25 µg/mL) – T5 (10 µg/mL). OPKA was statistically significant ($p < 0.05$ at a 95% confidence interval) at JG7 concentrations of 2.5 µg/mL ($p = 0.039$), 10 µg/mL ($p = 0.011$), 25 µg/mL ($p = 0.018$), and 50 µg/mL ($p = 0.009$). However, the change in cMtb CFUs relative to baseline, at the highest JG7 concentration of 100 µg/mL, was not statistically significant ($p = 0.460$). Moreover, there was a noticeable increase in bacterial CFUs at this concentration, relative to T2 - T7.

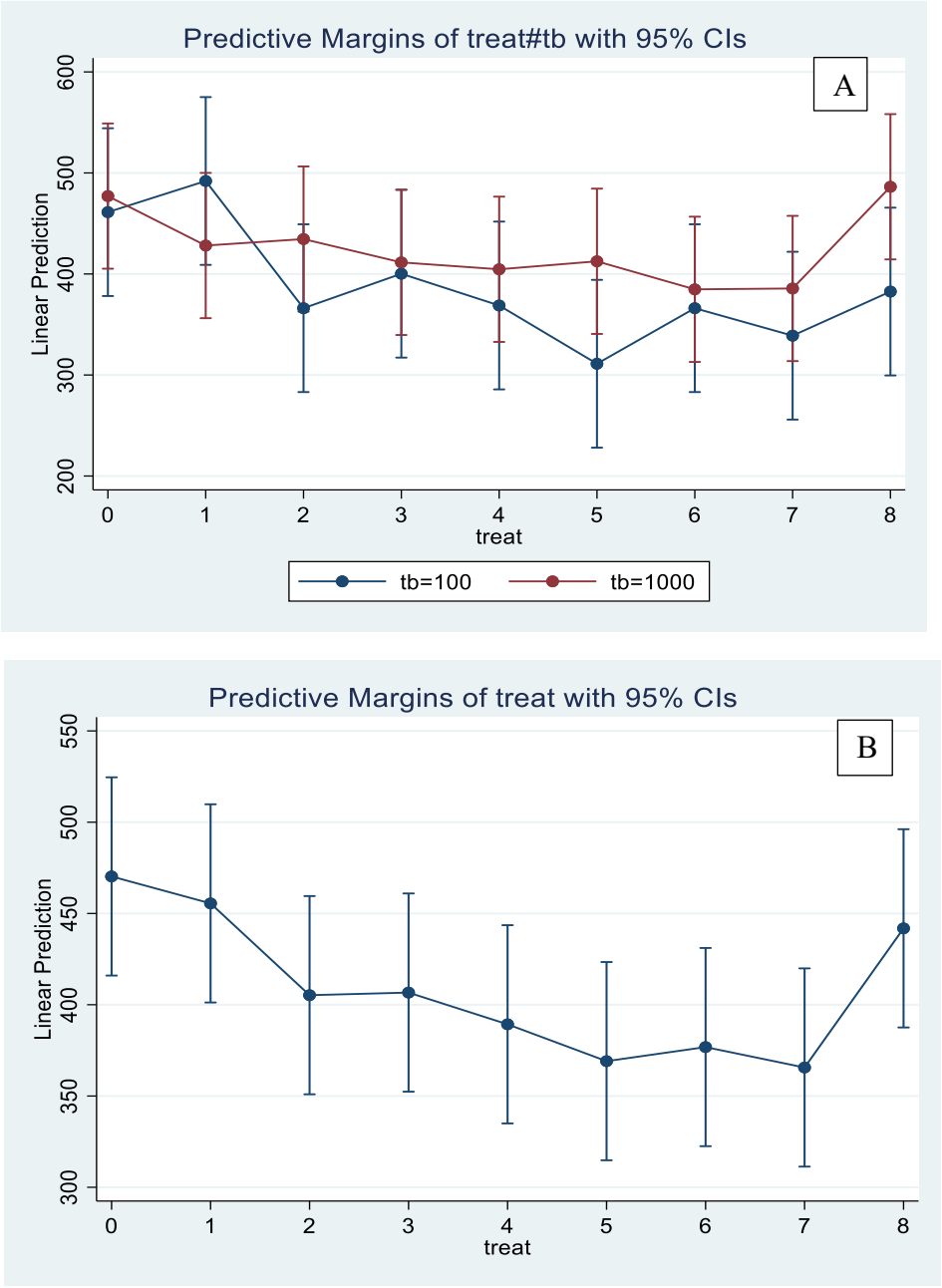


Figure 4.2: Predictive margins of change in cMtb CFUs by JG7 concentration for macrophage cell line U-937 and bacterial dilutions 1:100 and 1:1000 (A=Individually; B=Combined)
(treat 0 = baseline/control with no JG7, treat 1 = 0.25 µg/mL, treat 2 = 0.5 µg/mL, treat 3 = 1 µg/mL, treat 4 = 2.5 µg/mL, treat 5 = 10 µg/mL, treat 6 = 25 µg/mL, treat 7 = 50 µg/mL, treat 8 = 100 µg/mL)

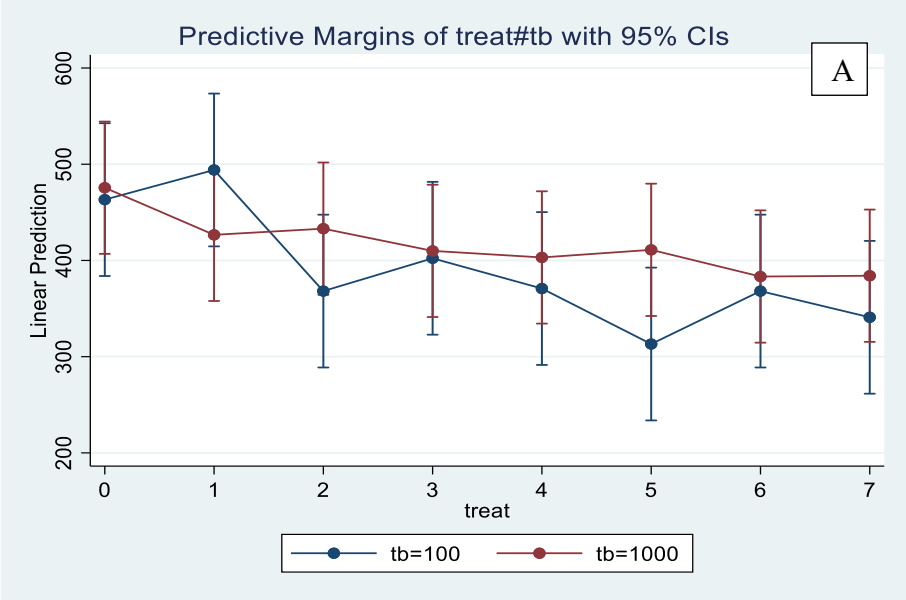
In **Table 4.4** above, statistically significant differences in OPKA were observed when the 1:100 bacterial dilution was compared with the 1:1000 (p = 0.048). However, when the data obtained for T8 (100 µg/mL) were excluded, the differences in OPKA for the 1:100 and 1:1000 dilutions were no longer significant (p = 0.176) (**Table 4.5**).

Table 4.5: Comparing cMtb dilutions 1:100 and 1:1000 (p-value, 0.176) by JG7 concentration (T0-T7) for effect on bacterial CFUs in U-937 macrophages (excluding T8)

Treatment*	Margin	t-value	95% CI	dy/dx	p-value
0	470.29	18.32	418.37 - 522.20		
1	455.52	17.75	403.61 - 507.44	-14.76	0.686
2	405.24	15.79	353.33 - 457.15	-65.05	0.081
3	406.67	15.85	354.75 - 458.58	-63.62	0.087
4	389.29	15.17	337.37 - 441.20	-81.00	0.031
5	369.10	14.38	317.18 - 421.01	-101.19	0.008
6	376.81	14.68	324.90 - 428.72	-93.48	0.014
7	365.62	14.25	313.71 - 417.53	-104.67	0.006

*Treatment=mAb JG7 concentration (µg/mL): T8=100; T7=50; T6=25; T5=10; T4=2.5; T3=1.0; T2=0.5; T1=0.25; T0=0 (control).; CI=confidence interval

Figure 4.3 is a margins plot showing the effects T1 to T7 (i.e. without T8) have on bacterial CFUs relative to T0 at cMtb dilutions 1:100 and 1:1000. According to **Figure 4.3**, relative to baseline CFU values for cMtb without mAb exposure, and excluding T8 (100 µg/mL), OPKA was statistically significant (p < 0.05 at 95% confidence interval) at JG7 concentrations of 2.5 µg/mL (p = 0.031), 10 µg/mL (p = 0.008), 25 µg/mL (p = 0.014), and 50 µg/mL (p = 0.006).



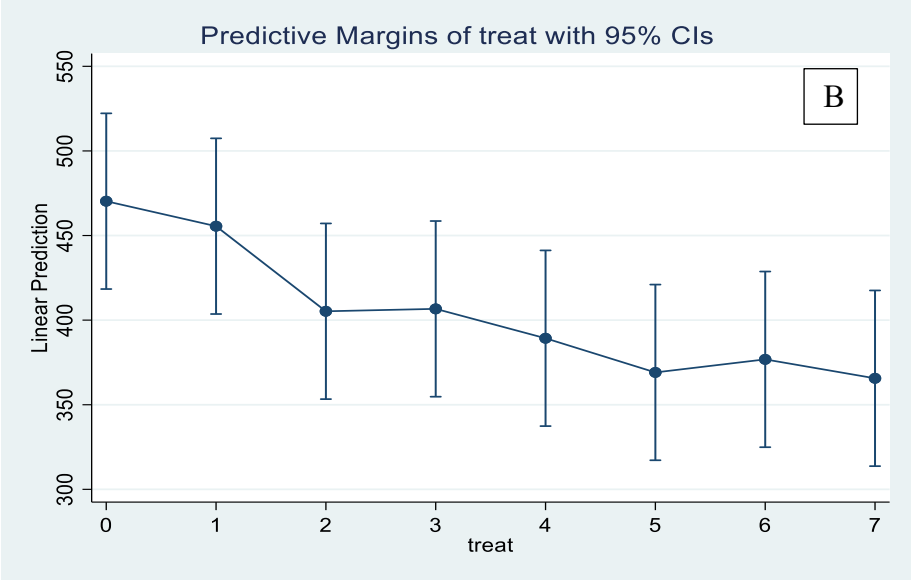


Figure 4.3: Predictive margins of change in cMtb CFUs by JG7 concentration (excluding treatment 8) for macrophage cell line U-937 and bacterial dilutions 1:100 and 1:1000 (A=Individually; B=Combined)

(treat 0 = baseline/control with no JG7, treat 1 = 0.25 µg/mL, treat 2 = 0.5 µg/mL, treat 3 = 1 µg/mL, treat 4 = 2.5 µg/mL, treat 5 = 10 µg/mL, treat 6 = 25 µg/mL, treat 7 = 50 µg/mL, treat 8 = 100 µg/mL)

4.5.2 Data obtained from OP assays with HL-60 granulocyte cell line

Like the U-937 macrophage cell line, the data sets obtained from OP assays for H37Ra and cMtb with HL-60 cells are summarized in **Table 4.6** (from line data shown in **Appendix C**, supplementary **Sheets 3-4**), which is a representative data set showing the average bacterial CFUs of the total number of observations in sample and control wells per bacterial dilution for each JG7 concentration used with the HL-60 cell line.

Table 4.6: Effect of JG7 at different concentrations on bacterial cells by strain type and bacterial dilution, in the HL-60 granulocyte cell line

Strain: H37Ra									
JG7 concentration (µg/mL)	Bacterial dilution**								
	1:100			1:1000			1:10 000		
	N	CFU		N	CFU		N	CFU	
		Mean	SD		Mean	SD		Mean	SD
0	12	474.7	152.6	24	394.5	174.2	12	449.9	99.0
0.25	6	459.2	105.9	12	415.8	187.5	6	326.5	135.9
0.5	6	391.7	67.5	12	357.3	275.7	6	280.5	80.8
1	6	260.5	116.5	12	338.5	190.9	6	293.8	110.5
2.5	6	380.7	170.7	12	416.8	158.8	6	404.5	161.3
10	6	485.3	106.5	12	414.9	221.1	6	334.2	125.7
25	6	463.2	145.1	12	496.3	248.2	6	587.2	246.7
50	6	281.8	110.8	12	313.7	166.2	6	261.5	145.4
100	6	325.8	188.4	12	301.9	110.2	6	361.5	122.0

Strain: cMtb									
JG7 concentration (µg/mL)	Bacterial dilution**								
	1:100			1:1000			1:10 000		
	N	CFU		N	CFU		N	CFU	
		Mean	SD		Mean	SD		Mean	SD
0	18	663.6	289.7	24	392.0	210.3	6	407.0	93.7
0.25	9	523.4	248.0	12	476.3	311.5	3	422.7	86.3
0.5	9	589.9	237.9	12	442.9	256.8	3	499.0	189.0
1	9	515.8	164.7	12	400.8	221.3	3	348.7	54.4
2.5	9	632.0	365.9	12	372.4	241.6	3	275.0	4.4
10	9	574.3	143.6	12	357.5	171.9	3	407.7	56.1
25	9	647.4	168.4	12	515.5	296.2	3	494.0	26.2
50	9	665.0	184.2	12	395.4	193.1	3	444.7	66.9
100	9	684.4	243.6	12	386.7	208.9	3	270.0	78.1

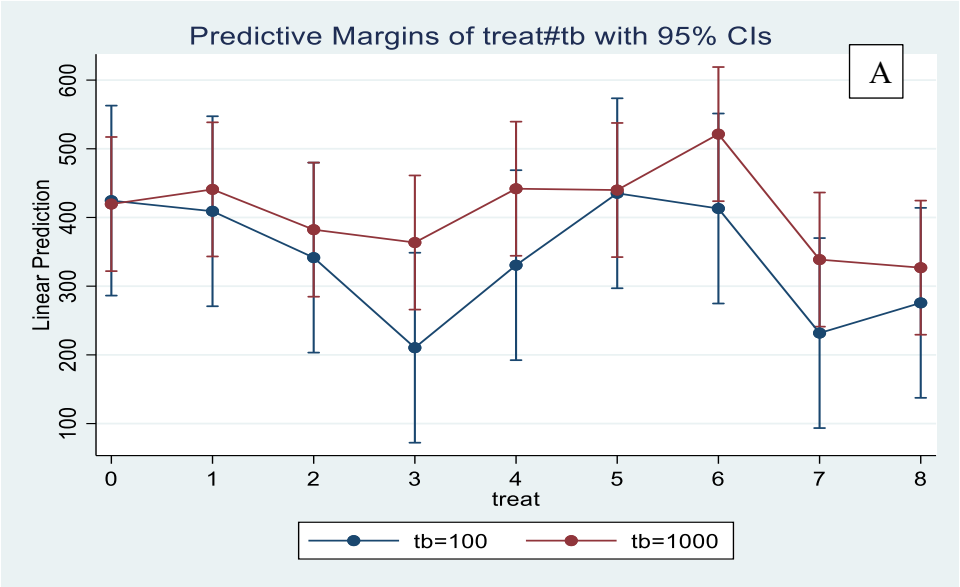
H37Ra (susceptible laboratory strain, ATCC 25177), cMtb (susceptible patient strain, clinical *M. tuberculosis*), N (number of observations), CFU (colony forming units), SD (standard deviation)
** Starting concentrations for dilution 1:100 were $1x10^8$ CFU/mL, $1x10^8$ CFU/mL or $1x10^9$ CFU/mL for dilution 1:1000, and $1x10^9$ CFU/mL for 1:10 000

Further statistical analysis was conducted on the data set obtained from OP assays for H37Ra and cMtb with HL-60 granulocyte cells as was performed with the data obtained for the U-937 macrophage cells (excluding the 1:10 000 dilution). **Figures 4.4** and **4.5** are margins plots drawn from **Tables 4.7** and **4.8** respectively, showing the effects that treatments 1 - 8 have on bacterial CFUs relative to treatment 0 for H37Ra and cMtb dilutions 1:100 and 1:1000.

Table 4.7: Comparing H37Ra dilutions 1:100 and 1:1000 (p-value, 0.026) by JG7 concentration (T0-T8) for effect on bacterial CFUs in HL-60 granulocyte cells

Treatment*	Margin	t-value	95% CI	dy/dx	p-value
0	421.22	10.74	341.63 - 500.81		
1	430.28	10.97	350.69 - 509.87	9.06	0.871
2	368.72	9.40	289.13 - 448.31	-52.50	0.350
3	312.50	7.97	232.91 - 392.09	-108.72	0.058
4	404.78	10.32	325.19 - 484.37	-16.44	0.769
5	438.39	11.18	358.80 - 517.98	17.17	0.759
6	485.28	12.38	405.69 - 564.87	64.06	0.256
7	303.06	7.73	223.46 - 382.65	-118.17	0.040
8	309.89	7.90	230.30 - 389.48	-111.33	0.052

* Treatment=mAb JG7 concentration (µg/mL): T8=100; T7=50; T6=25; T5=10; T4=2.5; T3=1.0; T2=0.5; T1=0.25; T0=0 (control).; CI=confidence interval



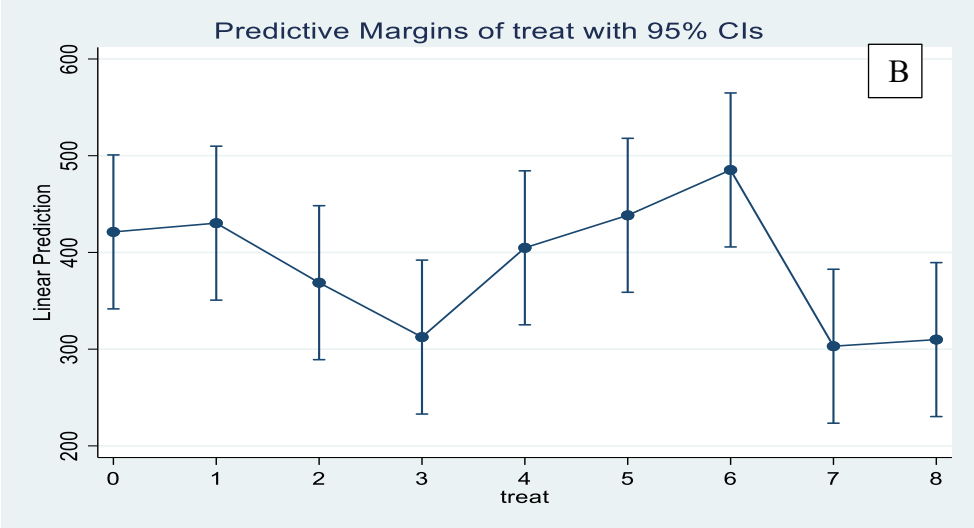


Figure 4.4: Predictive margins of change in H37Ra CFUs by JG7 concentration for granulocyte cell line HL-60 and bacterial dilutions 1:100 and 1:1000 (A=Individually; B=Combined)

(treat 0 = baseline/control with no JG7, treat 1 = 0.25 µg/mL, treat 2 = 0.5 µg/mL, treat 3 = 1 µg/mL, treat 4 = 2.5 µg/mL, treat 5 = 10 µg/mL, treat 6 = 25 µg/mL, treat 7 = 50 µg/mL, treat 8 = 100 µg/mL)

According to **Figure 4.4**, relative to baseline CFU values for H37Ra without mAb exposure, OPKA was only marginally significant ($0.05 \leq p \leq 1$, at a 95% confidence interval) at all eight JG7 concentrations. However, there is a noticeable decrease in bacterial CFUs at 1 µg/mL, 50 µg/mL, and 100 µg/mL, compared to baseline, and an increase in bacterial CFUs at 25 µg/mL compared to baseline, with statistically significant OPKA at 50 µg/mL ($p = 0.040$).

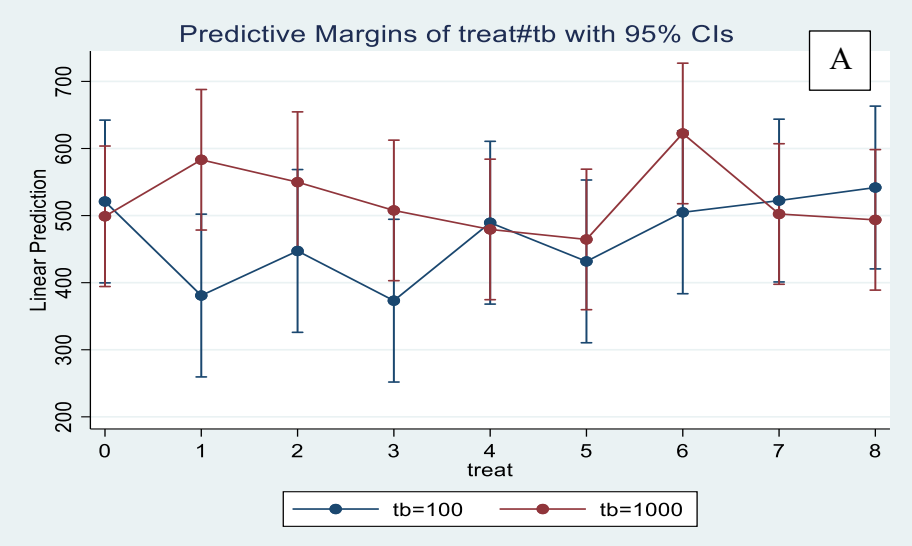
Analytical statistics making use of margins and linear prediction models as used in the sections above, are shown in **Table 4.8** for cMtb in HL-60 cells. **Figure 4.5** is a margins plot drawn from **Table 4.8**, showing the effects that treatments 1 to 8 (JG7 concentrations 0.25 µg/mL - 100 µg/mL respectively) have on bacterial CFUs relative to treatment 0 (control) at cMtb dilutions 1:100 and 1:1000.

According to **Figure 4.5**, relative to baseline CFU values for cMtb without mAb exposure, the bacterial CFUs seem relatively constant except at 1 µg/mL and 5 µg/mL (noticeable decrease in bacterial CFUs from baseline) and at 25 µg/mL (noticeable increase in bacterial CFUs from baseline).

Table 4.8: Comparing cMtb dilutions 1:100 and 1:1000 (p-value, 0.087) by JG7 concentration (T0-T8) for effect on bacterial CFUs in HL-60 granulocyte cells

Treatment*	Margin	t-value	95% CI	dy/dx	p-value
0	508.41	13.06	429.98 - 586.83		
1	496.48	12.76	418.05 - 574.90	-11.93	0.829
2	505.91	13.00	427.48 - 584.33	-2.50	0.964
3	450.05	11.57	371.62 - 528.47	-58.36	0.295
4	483.67	12.43	405.24 - 562.09	-24.74	0.655
5	450.43	11.58	372.00 - 528.85	-57.98	0.298
6	572.05	14.70	493.62 - 650.47	63.64	0.254
7	510.95	13.13	432.53 - 589.38	2.55	0.963
8	514.29	13.22	435.86 - 592.71	5.88	0.915

*Treatment=mAb JG7 concentration (µg/mL): T8=100; T7=50; T6=25; T5=10; T4=2.5; T3=1.0; T2=0.5; T1=0.25; T0=0 (control).; CI=confidence interval



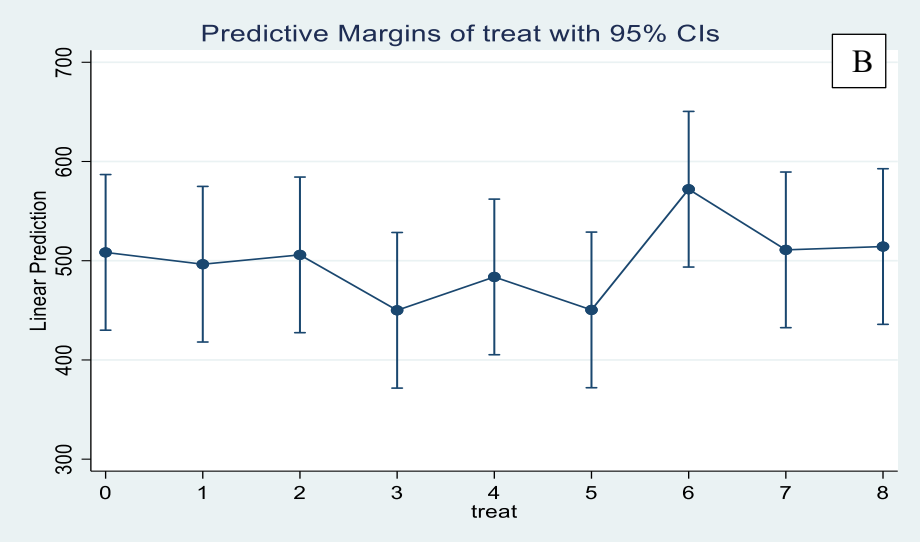


Figure 4.5: Predictive margins of change in cMtb CFUs by JG7 concentration for granulocyte cell line HL-60 and bacterial dilutions 1:100 and 1:1000 (A=Individually; B=Combined)

(treat 0 = baseline/control with no JG7, treat 1 = 0.25 µg/mL, treat 2 = 0.5 µg/mL, treat 3 = 1 µg/mL, treat 4 = 2.5 µg/mL, treat 5 = 10 µg/mL, treat 6 = 25 µg/mL, treat 7 = 50 µg/mL, treat 8 = 100 µg/mL)

4.6 Discussion

The aim was to assess the suitability of the novel mouse IgG1 mAb JG7 in enhancing the phagocytic and antimicrobial activities of human macrophage and granulocyte cell lines against susceptible *M. tuberculosis*. Three primary factors were considered in order to achieve this aim: strain type, mAb concentration, and bacterial dilution. Based on the first factor, strain type, two susceptible *M. tuberculosis* strains (H37Ra and cMtb) were investigated with each cell line.

Based on the data presented, mAb JG7 appears to affect the level of phagocytosis across all four groups investigated (two strain types each, in two cell lines). Generally, the bacterial CFU decreases in samples with JG7, relative to controls without JG7. A wide range of JG7 concentrations was used in order to determine the mAb concentration(s) needed for optimal opsonophagocytosis. In terms of OPKA, the lower JG7 concentrations (0.25 µg/mL and 0.5 µg/mL) did not seem effective, while the middle range of concentrations (1 µg/mL, 2.5 µg/mL, and 10 µg/mL) and the higher concentrations (50 µg/mL and 100 µg/mL) were marginally effective. These results are consistent with those in the study conducted by Sei and colleagues, who demonstrated that mAb-enhanced phagocytosis of *Mycobacterium smegmatis* in U-937 macrophage cells was statistically significant at higher mAb concentrations [Sei *et al*., 2019]. Overall, the 10 µg/mL concentration performed well with the U-937 cell line (Figures 4.1B and

4.2B) while the 1 µg/mL concentration performed well with the HL-60 cell line (Figures 4.4B, and 4.5B).

This novel mAb clearly plays a role in the phagocytosis of *M. tuberculosis*, although, with the trends observed from the results obtained, whereby, fluctuations in OPKA are observed across different JG7 concentrations (OPKA increases over a range of JG7 concentrations followed by a decrease over higher JG7 concentrations, followed by another increase at even higher JG7 concentrations and so on [this trend is more frequent in H37Ra than in cMtb]), the significance of this role warrants further investigation. However, one could argue that there is an optimum point for opsonophagocytosis to take place, whereby the number of *M. tuberculosis* bacilli corresponds to the number of human phagocyte cells at a given JG7 concentration. A study by Bangani *et al*., (2016) noted lower levels of opsonophagocytosis when 50% of IgG-containing serum was used compared to 25%. One of the major factors which influence the choice of an appropriate HDT is concentration. An intervention, in which lower concentrations are optimal for enhancing bacterial clearance, is beneficial to the patient since it limits concerns with regards to toxicity.

Opsonophagocytic killing activity was more effective at the lower bacterial dilution of 1:100 (more bacteria) compared to the higher bacterial dilution of 1:1000 (less bacteria) for both H37Ra and cMtb in both human cell lines (Figures 4.2A, 4.3A, 4.4A, and 4.5A) although this difference was only marginally significant in some cases. However, statistically significant differences between OPKA at a 1:100 and a 1:1000 bacterial dilution were observed for H37Ra with the HL-60 cell line ($p = 0.026$) (Figure 4.4A) and for cMtb with the U-937 cell line ($p = 0.048$) (Figure 4.2A). This points towards an increase in opsonophagocytosis with increased bacterial uptake by the phagocyte cells. Although the reason for this is not understood, it is interesting to find a potential intervention that acts better at higher bacterial concentrations. Bacterial dilution is a crucial factor as it translates to bacterial load in the patient, and an HDT in which low doses of the intervention can enhance the clearance of high bacterial loads is desirable.

Worthy of note is the fact that some secondary factors were also assessed, but due to the limited number of observations per factor, no conclusions can be drawn based on the results obtained. To this effect, two bacterial growth phases (mid-logarithmic and stationary phases) were used, since *M. tuberculosis* populations display different characteristics at different phases of their growth. Most investigations use mid-logarithmic phase bacteria because *M. tuberculosis* populations are generally metabolically active at this stage [Krishnan *et al*., 2013, Shekhawat *et*

al., 2014, Bangani *et al*., 2016, Liu *et al*., 2018]. Stationary phase *M. tuberculosis* on the other hand, contains sub-populations of both metabolically active and inactive (persisters) bacteria, which may become reactivated at a later stage [Zhao *et al*., 2015, Rosser *et al*., 2017, Vulin *et al*., 2018]. It is therefore important that a potential therapeutic intervention be able to enhance the clearance of different *M. tuberculosis* populations. Although there was no statistical comparison for mid-logarithmic versus stationary phase bacteria due to the limited number of experiments carried out with the stationary phase bacteria, there seemed to be no obvious differences in OPKA with both human cell lines used.

Two cell differentiation times (72 and 96 hours for U-937 cells, 120 and 144 hours for HL-60 cells) were tested in preparation for the OP assays. Higher OPKA was observed at the earlier differentiation times of 72 hours and 120 hours for U-937 and HL-60 cells respectively. These findings are consistent with those by Sei *et al*., (2019) who show statistically significant OPKA at lower differentiation times, with both *Mycobacterium smegmatis* and *M. tuberculosis*. The reason(s) for this difference is unclear, although, one might speculate that since these cells are differentiated with toxic agents such as PMA and DMSO which in turn induce their mortality, longer periods of differentiation would imply a decrease in cell viability and function.

Two plate incubation times (4 and 48 hours) were tested, and there seemed to be no obvious differences in OPKA at 4 hours and 48 hours of incubation. This is a favourable outcome, since human phagocytes not only form part of the innate immune system but are the first responders during *M. tuberculosis* infection [Schafer *et al*., 2009, Ahmad, 2011]. Phagocytic activity within 4 hours implies they are recruited in the first few hours following infection, while phagocytic activity at up to 48 hours implies that since these cells serve as a link between the host's innate and adaptive immune systems, they continue to recruit other components of host immunity long after initial infection [Walzl *et al*., 2011, Aberdein *et al*., 2013, Alvarez-Jiménez *et al*., 2018].

Overall, the U-937 cell line performed better on cMtb than H37Ra (Figure 4.3 versus 4.2) while the HL-60 cell line performed better on H37Ra than cMtb (Figure 4.4 versus 4.5) although the U-937 cell line performed better than the HL-60 cell line, overall. This strain preference is probably due to different uptake abilities by each cell line as a result of their different strain affinities. The higher performance of the U-937 cell line compared to the HL-60 cell line is to be expected, because macrophages are commonly used in *M. tuberculosis* studies since these are the first immune cells to be mobilised by the human innate immune system during *M. tuberculosis* infection [Sequeira *et al*., 2018, Kawahara *et al*., 2019].

In summary, opsonophagocytosis was observed at different concentrations of both susceptible laboratory strain H37Ra and patient strain cMtb, in both human U-937 macrophage and HL-60 granulocyte cell lines, even at different bacterial growth phases. These findings are similar to those obtained in studies carried out by Chen and colleagues, where the opsonization of *M. tuberculosis* was proportional to increases in IgG response [Chen *et al.*, 2016]. In addition, phagocytosis, phagolysosomal fusion, and intracellular growth inhibition were significantly enhanced upon opsonization, and these enhancements correlated with an increase in IgG titres [Chen *et al.*, 2016]. The present study had a few limitations, from the exclusion of the data obtained for the 1:10 000 bacterial dilution as well as the absence of any kind of comparative analysis for the secondary factors investigated, due to the relatively small number of observations obtained, to the lack of any background information on the cMtb strain obtained which might give some indication on the reason why it exhibits different characteristics to the H37Ra laboratory strain used. Further investigations on different *M. tuberculosis* strains from different patients, are necessary to confirm the findings obtained in this study, since *M. tuberculosis* strains from different individuals could display unique characteristics that may influence their opsonophagocytosis within human phagocytes.

4.7 Conclusion

Novel IgG1 mouse mAb JG7-enhanced phagocytosis of live susceptible *M. tuberculosis* strains using U-937 and HL-60 human cells, was demonstrated through several *in vitro* OP assays. The OPKA was measured from experimental controls, based on bacterial CFU counts. JG7 binds to all forms of *M. tuberculosis* as shown previously and enhances phagocytosis of both active and stationary *M. tuberculosis* in macrophages and granulocytes. The results obtained from this study will hopefully translate to the enhancement and clearance of both latent and active *M. tuberculosis* when tested *in vivo*. Both binding and killing data suggest that opsonic mAbs, directed against *M. tuberculosis*, may be valuable as HDTs against TB.

Ethics approval

None of the procedures performed involved human participants. Ethical clearance for the study was obtained from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria (reference number-487/2018).

4.8 References

Aberdein JD, Cole J, Bewley MA, Marriott HM, Dockrell DH (2013) Alveolar macrophages in pulmonary host defence the unrecognized role of apoptosis as a mechanism of intracellular bacterial killing. *Clinical and Experimental Immunology* **174**(2): 193–202.

Achkar JM, Casadevall A (2013) Antibody-mediated immunity against tuberculosis: Implications for vaccine development. *National Institute of Health-Author's manuscript* **13**(3): 250–262.

Alvarez-Jiménez DV, Leyva-Paredes K, García-Martínez M, Vázquez-Flores L, García-Paredes GV, Campillo-Navarro M, Romo-Cruz I, Rosales-García HV, Castañeda-Casimiro J, González-Pozos S, Hernández MJ, Wong-Baeza C, García-Pérez EB, Ortiz-Navarrete V, Estrada-Parra S, Serafín-López J, Wong-Baeza I, Chacón-Salinas R, Estrada-García I (2018) Extracellular vesicles released from *Mycobacterium tuberculosis*-infected neutrophils promote macrophage autophagy and decrease intracellular mycobacterial survival. *Frontiers in Immunology* **9**: 272.

Balu S, Reljic R, Lewis ML, Pleass RJ, McIntosh R, van Kooten C, van Egmond M, Challacombe S, Woof JM, Ivanyi J (2011) A novel human IgA monoclonal antibody protects against tuberculosis. *Journal of Immunology* doi: 10.4049/jimmunol.1003189.

Bangani N, Nakiwala J, Martineau RA, Wilkinson JR, Wilkinson AK, Lowe MD (2016) HIV-1 Infection Impairs CD16 and CD35 Mediated Opsonophagocytosis of *Mycobacterium tuberculosis* by Human Neutrophils. *Journal of Acquired Immune Deficiency Syndrome* **73**: 263–267.

Chen T, Blanc C, Eder ZA, Prados-Rosales R, Souza OCA, Kim SR, Glatman-Freedman A, Joe M, Bai Y, Lowary LT, Tanner R, Brennan JM, Fletcher AH, McShane H, Casadevall A, Achkar MJ (2016) Association of Human Antibodies to Arabinomannan With Enhanced Mycobacterial Opsonophagocytosis and Intracellular Growth Reduction. *Journal of Infectious Diseases* **214**: 300–10.

Colangeli R, Jedrey H, Kim S, Connell R, Ma S, Chippada Venkata DU, Chakravorty S, Gupta A, Sizemore EE, Diem L, Sherman RD, Okwera A, Dietze R, Boom HW, Johnson LJ, MacKenzie RW, Alland D for the DMID 01-009/Tuberculosis Trials Consortium Study 22 Teams (2018) Bacterial Factors That Predict Relapse after Tuberculosis Therapy. *New England Journal of Medicine* **379**: 823–33.

Kawahara YJ, Irvine BE, Alter G (2019) A Case for Antibodies as Mechanistic Correlates of Immunity in Tuberculosis. *Frontiers in Immunology* doi: 10.3389/fimmu.2019.00996.

Krishnan N, Robertson DB, Thwaites G (2013) Pathways of IL-1b secretion by macrophages infected with clinical *Mycobacterium tuberculosis* strains. *Tuberculosis* **93**: 538–547.

Liu F, Chen J, Wang P, Li H, Zhou Y, Liu H, Liu Z, Zheng R, Wang L, Yang H, Cui Z, Wang F, Huang X, Wang J, Sha W, Xiao H, Ge B (2018) MicroRNA-27a controls the intracellular survival of *Mycobacterium tuberculosis* by regulating calcium associated autophagy. *Nature Communications* doi: 10.1038/s41467-018-06836-4.

Mittar D, Paramban R, McIntyre C (2011) Flow Cytometry and High-Content Imaging to Identify Markers of Monocyte-Macrophage Differentiation. *BD Biosciences Application note* pg 1–20.

Rekha SR, Mily A, Sultana T, Haq A, Ahmed S, Kamal MSM, van Schadewijk A, Hiemstra SP, Gudmundsson HG, Agerberth B, Raqibet R (2018) Immune responses in the treatment of drug-sensitive pulmonary tuberculosis with phenylbutyrate and vitamin D3 as host directed therapy. *BMC Infectious Diseases* **18**: 303.

Romagnoli A, Petruccioli E, Palucci I, Camassa S, Carata E, Petrone L, Mariano S, Sali M, Dini L, Girardi E, Delogu G, Goletti D, MG Fimia (2018) Clinical isolates of the modern *Mycobacterium tuberculosis* lineage 4 evade host defense in human macrophages through eluding IL-1β-induced autophagy. *Cell Death and Disease* **9**: 624.

Romanowski K, Balshaw FR, Benedetti A, Campbell RJ, Menzies D, Khan AF, Johnston CJ (2018) Predicting tuberculosis relapse in patients treated with the standard 6-month regimen: an individual patient data meta-analysis. *BMJ Journals* doi: http://dx.doi.org/10.1136/thoraxjnl-2017-211120.

Rosser A, Stover C, Pareek M, Mukamolova VG (2017) Resuscitation-promoting factors are important determinants of the pathophysiology in *Mycobacterium tuberculosis* infection. *Critical Reviews in Microbiology* **43**(5): 621–630.

Roy E, Lowire DB, Jolles SR (2007) Current strategies in TB immunotherapy. *Current Molecular Medicine* **77**: 373–38.

Sei JC, Shey B, Schuman FR, Rikhi N, Muema K, Rodriguez DJ, Daum TL, Fourie PB, Fischer WG (2019) Opsonic Monoclonal Antibodies Enhance Phagocytic Killing Activity and Clearance of *Mycobacterium tuberculosis* from Blood in a Quantitative qPCR Mouse Model. *Heliyon* **5**(9): 1–10.

Shekhawat DS, Jain KR, Gaherwar MH, Purohit JH, Taori MG, Daginawala FH, Kashyap SR (2014) Heat shock proteins: Possible biomarkers in pulmonary and extrapulmonary tuberculosis. *Human Immunology* **75**: 151–158.

Siqueira da Silva M, Ribeiro de Moraes R, Travassos HL (2018) Autophagy and its interaction with intracellular Bacterial Pathogens. *Frontiers in Immunology* **9**(935): 1–7.

Vulin C, Leimer N, Huemer M, Ackermann M, Zinkernagel SA (2018) Prolonged bacterial lag time results in small colony variants that represent a sub-population of persisters. *Nature Communications* doi: 10.1038/s41467-018-06527-0.

Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A (2011) Immunological biomarkers of tuberculosis. *Nature Reviews in Immunol*ogy **11**: 343–354.

Welch RJ, Lawless KM, Litwin CM (2012) Antituberculosis IgG Antibodies as a Marker of Active *Mycobacterium tuberculosis* Disease. *Journal of Clinical and Vaccine Immunology* **19**(4): 522–526.

Woo M, Wood C, Kwon D, Park KH, Fejer G, Delorme V (2018) *Mycobacterium tuberculosis* Infection and Innate Responses in a New Model of Lung Alveolar Macrophages. *Frontiers in Immunology* **9**: 438.

Zhao S, Song X, Zhao Y, Qiu Y, Mao F, Zhang C, Bai B, Zhang H, Wu S, Shi C (2015) Protective and therapeutic effects of the resuscitation-promoting factor domain and its mutants against *Mycobacterium tuberculosis* in mice. *FEMS Pathogens and Disease* **73**: 3.

Zimmermann N, Thormann V, Hu B, Köhler A, Imai-Matsushima A, Locht C, Arnett E, Schlesinger SL, Zoller T, Schürmann M, Kaufmann HES, Wardemann H (2016) Human isotype-dependent inhibitory antibody responses against *Mycobacterium tuberculosis*. *EMBO Molecular Medicine* **8**: 1325–1339.

Zumla A, Nahid P, Cole ST (2013) Advances in the development of new tuberculosis drugs and treatment regimens. *Nature Reviews Drug Discovery* **12**: 388–404.

Zumla A, Chakaya J, Centis R, D'Ambrosio L, Mwaba P, Bates M, Kapata N, Nyirenda T, Chanda D, Mfinanga S, Hoelscher M, Maeurer M, Migliori GB (2015) Tuberculosis treatment and management--an update on treatment regimens, trials, new drugs, and adjunct therapies. *Lancet Respiratory Medicine* **3**(3): 220–34.

Chapter 5

5 Cytokine response to monoclonal antibody-enhanced phagocytosis of *Mycobacterium tuberculosis* in human granulocyte and mononuclear cells

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5.1 Overview

Summary: The cytokines produced during a two-way interaction between human phagocytes and *Mycobacterium tuberculosis* (*M. tuberculosis*) have been well characterized. This chapter presents data to evaluate the cytokine response during monoclonal antibody (mAb)-enhanced phagocytosis. Chapter 5 presents findings on the cytokine response to unstimulated, lipopolysaccharide stimulated, *M. tuberculosis* activated, and *M. tuberculosis* plus mAb activated human granulocyte and mononuclear cells. The complete experimental procedures and supplementary data for this chapter are provided in Appendices A, B, and D.

Contribution to the thesis and novelty: Findings are presented on the cytokines produced when isolated human granulocytes and mononuclear cells interact either with lipopolysaccharide, *M. tuberculosis*, or *M. tuberculosis* and the mAb. Comparisons between each of these interactions have been performed, in order to determine if the variations in cytokine expression are significant from one interaction to the next, and the findings have been presented here.

Contributions of Candidate: The candidate was involved with all experimental procedures from cell isolation, to bacterial infection, to the multiplex suspension assays performed at UP. The candidate was responsible for interpreting the analysed data obtained in line with the stated objectives of the overall thesis.

Publication status: Not published as at the time of preparation of this chapter. Paper to be submitted to *Cytokine* for publication.

5.2 Abstract

Introduction: The cytokine response is part of a complex system involving a variety of proteins or peptides, acting as regulators of processes which influence immune responses such as inflammation. Cytokines play an important role in the host's response to infection and mediate the interaction between phagocytic cells and *Mycobacterium tuberculosis* (*M. tuberculosis*). There is, however, limited information about the effect of monoclonal antibodies (mAbs) on these interactions. The cytokines expressed during such an interaction may lead to knowledge about what is beneficial to the host in order to inform antibody-mediated host-directed therapies.

Methods: The aim of this investigation was to assess the cytokine response to granulocyte/mononuclear (MNL) cell - mAb - *M. tuberculosis* interactions after mAb-enhanced phagocytosis (opsonophagocytosis). Blood was collected from four healthy volunteers and granulocytes and MNL cells were isolated from each participant (labelled groups 1 - 4). The isolated cells from each individual represented one assay consisting of a sub-set of four groups or interactions (labelled sub-groups 1 - 4). Flow cytometry was performed to assess the purity and percentage viability of granulocyte or MNL cell populations in the blood and a bead-based multiplex suspension array assay was then used to measure cytokine expression in granulocytes or MNL cells either, unexposed as a negative control (sub-group 1), exposed to *M. tuberculosis* H37Rv (ATCC 26518) (sub-group 2), exposed to both H37Rv and novel IgG1 mAb JG7 (sub-group 3), or exposed to lipopolysaccharide (LPS) as a positive control (sub-group 4), for 24 hours. Cytokine levels were measured for each of these sub-groups per group, using a standard human cytokine panel of seven cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70, IFN-γ and TNF-α).

Results: Flow cytometry showed cell viability ranging between 80% and 93% for the granulocytes and between 63% and 87% for the MNL cells for the four donor groups used. Flow cytometry also confirmed the presence of CD14+ monocyte populations, CD3+ lymphocyte populations, alongside CD4+ and CD8+ T-cell populations in the isolated MNL cells. Comparing the difference in cytokine production between sub-groups, with the granulocytes, levels of IL-1β and TNF-α were significantly higher in sub-groups 2 and 3 when compared to the negative control, and levels of IL-6, IL-10 and IL-12p70 were lower in sub-groups 2 and 3 when compared to the positive control. While significantly higher IL-8 levels were observed in sub-group 3 versus the negative control ($p = 0.0466$), this was not the case for sub-group 2 ($p = 0.4365$). With MNL cells, higher levels of all the cytokines, except IL-12p70 (which was only higher in sub-

group 2), were observed in sub-groups 2 and 3 when compared with the negative control. Higher levels of IL-1β, IFN-γ, and TNF-α, and lower levels of IL-10, were observed in sub-groups 2 and 3 when compared to the positive control. The addition of the mAb did not appear to have a significant impact on the expression levels of the cytokines tested. There was a large, but non-significant, increase in IL-8 expression by the neutrophils exposed to the mAb, which is consistent with the increased opsonophagocytosis observed previously (chapter 4). In contrast, the mAb-exposed MNL cells expressed significantly lower levels of IL-12p70, which, seen in conjunction with the increase in IL-10 in this sub-group, could point towards attenuation of pro-inflammatory responses by the mAb.

Conclusion: The novel IgG1 mAb used in this study does not seem to have played a significant role in cytokine over- or under-production, which *in vivo*, could be quite detrimental to the host. However, a cautious interpretation of the results does suggest that the mAb increased the uptake of mycobacteria by neutrophils, possibly manifesting as a pro-inflammatory effect in these cells, while effecting a significant anti-inflammatory response in MNL cells. With the limited data produced, a larger cohort is needed in order to fully assess the effect this mAb has on cytokine expression.

Keywords: cytokine, interleukin, granulocyte, mononuclear cell (MNL), *M. tuberculosis,* monoclonal antibody (mAb).

5.3 Introduction

Cytokines are molecular proteins or peptides that mediate physiological processes such as intercellular communication in the immune system. They play an important role in the host's response to infection and mediate the interaction between immune cells and invading pathogens, such as *Mycobacterium tuberculosis* (*M. tuberculosis*). The host's innate immune response and resistance against *M. tuberculosis* infection has been well characterised [Cavalcanti *et al*., 2012]. Macrophages (differentiated monocytes) and granulocytes (predominantly neutrophils) are the first immune cells that have contact with *M. tuberculosis* in lung alveoli [Walzl *et al*., 2011]. Macrophages interact with *M. tuberculosis* via numerous receptors, such as toll-like receptors (TLRs), which can induce distinct innate immune responses that may eliminate the bacterium, even before the establishment of adaptive immunity [Walzl *et al*., 2011; Aberdein *et al*., 2013; Alvarez-Jiménez *et al*., 2018].

The binding of these TLRs to their ligands induces the phagocytosis of *M. tuberculosis* and the production of pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), and the interleukins (IL), notably IL-1β, IL-6, IL-8, and IL-12p70 which initiate and maintain cell inflammation, as well as promote the activation and migration of other immune cells to the site of infection [D'Avila *et al*., 2008; Lin *et al*., 2010; Weiss and Schaible, 2015]. An unchecked pro-inflammatory response can induce damage to host tissue and needs to be counteracted by an appropriate anti-inflammatory response driven by various cytokines including IL-4, IL-10, and transforming growth factor beta-β [Domingo-Gonzalez *et al*., 2016; Lyadova, 2017; Gideon *et al*., 2019]. A carefully balanced response is needed to ensure adequate antimicrobial activity while preventing uncontrolled damage to host tissue.

Granulocytes are the most abundant human phagocytes, playing a significant role in protection against *M. tuberculosis.* Not only are they involved in the phagocytosis and killing of microorganisms through several mechanisms, including the lytic enzymes and antimicrobial peptides found in their lysosomes, and their production of reactive oxygen species, but they also act as antigen-presenting cells by secreting extracellular vesicles (EVs), which help present TB to macrophages for phagocytosis [Lyadova, 2017; Alvarez-Jiménez *et al*., 2018]. This is demonstrated by the fact that granulocyte EVs infected with the *M. tuberculosis* laboratory strain H37Rv (EV-TB) reduce the amount of *M. tuberculosis* in human macrophages by increasing the superoxide anion production in these macrophages [Alvarez-Jiménez *et al*., 2018]. It is noteworthy that both superoxide anion production and TLR 2/6 ligation are thought to induce the more complex process of autophagy [Alvarez-Jiménez *et al*., 2018].

M. tuberculosis manages to evade these innate immune responses championed by human macrophages and granulocytes via numerous mechanisms, including the production of antigenic peptides such as culture filtrate protein-10 (CFP-10) and early secretory antigenic target-6 (ESAT-6), that delay the migration of effector T-cells to the site of infection, thereby, allowing the bacteria to replicate (Krishnan *et al*., 2013; Deretic, 2014). This leads to a delay in the induction of the adaptive immune response which involves antibody-mediated immunity [Walzl *et al*., 2011]. Our knowledge of the protective role of antibody-mediated immunity in tuberculosis (TB) pathology remains incomplete, even though antibody-producing B cells are found in substantial numbers in granulomas [Walzl *et al*., 2011]. Studies of *M. tuberculosis* infection in mouse lungs suggested that B cells regulate the host's neutrophilic response, including the level of neutrophil infiltration and granulomatous inflammation, upon exposure to mycobacteria [Kozakiewicz *et al*., 2013]. In the same light, the effect a monoclonal antibody

(mAb) intervention has on a phagocyte - *M. tuberculosis* interaction is relatively unknown; information about how this alters the dynamic with regards to cytokine expression is essential for informing antibody-mediated host-directed therapies (HDTs). A mAb which produces a cytokine response that suppresses inflammation (anti-inflammatory) would be more beneficial to the host, as a therapeutic intervention since it would minimize host tissue damage.

5.4 Materials and methods

5.4.1 Monoclonal antibody JG7

A novel mouse IgG1 mAb, JG7 III D3 I F9 (JG7), selected from a broad panel of novel mAbs (Longhorn Vaccines and Diagnostics, unpublished data) was previously evaluated [Sei *et al.*, 2019], and it was demonstrated that this mAb had the ability to bind to both susceptible and resistant *M. tuberculosis* strains at different concentrations. As described in Chapters 3 and 4, opsonophagocytic killing of *M. tuberculosis* in the presence of JG7 was also demonstrated. The specific antigenic epitopes or binding receptors were not specifically identified, but there is suspicion that JG7 binds to an epitope involving *M. tuberculosis* peptidoglycan.

5.4.2 Sample preparation for flow cytometry and opsonophagocytic assay

Collection of blood from healthy human volunteers: A volume of approximately 100 mL of heparinised venous blood (5 units of preservative-free heparin per mL) was collected from each of four non-smoking, healthy adult volunteers. All blood donors gave written informed consent. All donors underwent a health screen consisting of a blood pressure check and an overall health assessment (by means of completing a questionnaire) by a qualified nursing sister.

Isolation of granulocytes from human blood: Granulocytes were isolated by centrifugation (Beckman Coulter, Indiana, USA) on Histopaque-1077 (Sigma-Aldrich GmbH, Munich, Germany) cushions at 400 xg for 25 minutes at room temperature. Following centrifugation, the granulocyte layer was removed into 50 mL falcon tubes (Thermo Fisher Scientific, Massachusetts, USA). Pre-prepared 3% gelatine solution (Merck KGaA, Darmstadt, Germany) in phosphate-buffered saline (PBS, pH 7.4) (Merck KGaA, Darmstadt, Germany) was added to the collected granulocyte layer, mixed well and incubated (ESCO Technologies, Missouri, USA) for 15 minutes at 37ºC. The leukocyte-rich supernatant was then collected into clean 50 mL conical tubes and centrifuged at 300 xg for 10 minutes at 4ºC. The supernatant was then discarded, and 0.83% ammonium chloride solution was added to the tubes to lyse any

contaminating erythrocytes. The tubes were inverted several times to ensure thorough mixing and left on ice for a further 10 minutes. After incubating on ice, the tubes were centrifuged at 300 xg for 10 minutes at 4ºC and the resultant pellet containing granulocytes (including neutrophils which are the most abundant) was washed with cold PBS by centrifugation at 280 xg for 10 minutes at 4ºC. The cells were re-suspended in a small volume of RPMI 1640 medium (Sigma-Aldrich GmbH, Munich, Germany) to facilitate enumeration.

Isolation of mononuclear cells from human blood: The peripheral blood mononuclear (MNL) cells were isolated by centrifugation on Histopaque-1077 cushions at 400 xg for 25 minutes at room temperature. Following centrifugation, the MNL layer was removed into 50 mL falcon tubes. The cells were washed (1/5 dilution) with PBS containing 250 µM of ethylene glycol tetra-acetic acid (pH 7.4; Sigma-Aldrich GmbH, Munich, Germany). The cell suspension was then centrifuged at 300 xg for 10 minutes at 4ºC. The cell pellet, which consisted predominantly of MNLs, contaminating erythrocytes and granulocytes, was re-suspended in 20 mL of sterile, ice-cold, 0.83% ammonium chloride and kept on ice for 10 minutes in order to remove the contaminating erythrocytes, followed by an additional centrifugation at 300 xg for 10 minutes at 4ºC. The cell pellet was re-suspended in RPMI 1640 medium to facilitate enumeration.

***Cell counting*:** Granulocytes and MNLs were enumerated microscopically using a haemocytometer (Lasec, Cape Town, South Africa), by adding 50 µL of each cell suspension to 450 µL of Gentian Violet solution (counting fluid) and loading 20 µL of the mixture unto the haemocytometer. Counting was performed using the x40 objective of the microscope (Reichert Jung Microstar, New York, USA) and the cell suspension was then made up to a final concentration of 10^7 cells/mL.

Preparation of heat-inactivated serum from human blood: A volume of 10 mL of blood was collected from each blood donor in red-top vacutainers (Becton, Dickinson, New Jersey, USA) with no anti-coagulant for the isolation of serum. The serum was drawn off following clotting at 37ºC for 60 minutes and centrifugation at 300 xg (3000 rpm) for 10 minutes at room temperature, followed by heating at 56ºC for 30 minutes, in order to ensure inactivation of complement. The serum was then stored at room temperature until use.

5.4.3 Flow cytometry

Cell viability: A volume of 50 µL of each sample containing the isolated granulocytes or MNL cells was aliquoted into flow tubes (Becton Dickinson, New Jersey, USA). A 1 mL volume of

Propidium Iodide (Beckman Coulter, California, USA) was added into each flow tube and the samples were run on the CytoFLEX S cytometer (Beckman Coulter, California, USA) using the appropriate protocol for each cell type. Cell viability was calculated in percentages.

Identification of different cell types in mononuclear cell population: A volume of 50 µL of sample containing the isolated MNL cells was aliquoted into each flow tube. A 5 µL volume of each antibody (CD4-AF 700 [Becton Dickinson, New Jersey, USA], CD3-FITC [Becton Dickinson], CD8-PE [Beckman Coulter, California, USA], CD14-ECD [Beckman Coulter]) and 30 µL of PBS were added to each tube. The tube was incubated in the dark for 15 minutes. A 1 mL volume of PBS was added, and the tube centrifuged at 500 xg for 5 minutes at room temperature. The supernatant was discarded, and the pellet re-suspended in 1 mL of PBS. The sample was then run on the CytoFLEX S cytometer, using the appropriate protocol.

Flow cytometry data analysis: Granulocyte and MNL cell populations were gated by placing regions around aggregated cells according to the appropriate protocols for quantification. Histograms were then plotted based on the percentage and number of positive cells for each marker used.

5.4.4 Bacterial preparation for opsonophagocytic assay

An inoculum of *M. tuberculosis* strain H37Rv ATCC 26518 was prepared in Middlebrook 7H9 broth (Becton Dickinson, New Jersey, USA) by adding approximately 10^5 colony forming units (CFU)/mL of the bacteria into 7H9 broth and growing the cultures at 37ºC in a shaking incubator (Labotec, Midrand, South Africa) until an optical density (OD) of 0.6 - 1.0 was reached at 540 nm, using a PowerWaveX spectrophotometer (BioTek Instruments Inc., Vermont, USA). The culture was centrifuged at 480 xg for 10 minutes at room temperature. The supernatant was discarded, and the pellet washed once with 50 mL PBS (pH 7.4). Approximately 5 mL volumes of glass beads (3 mm) were added to the pellets and vortexed to disperse the clumps, and the bacterial cells re-suspended in sterile PBS. The cell suspension density was adjusted to an OD of 1.2 at 540 nm (approximately 10^8 CFU/mL) as determined by colony-counting procedures described by Mothiba *et al*. (2015). Briefly, 10-fold serial dilutions of the bacteria were made in PBS, followed by plating 100 µL of each dilution on 7H10 agar medium (Becton Dickinson, New Jersey, USA).

The plate cultures were incubated for three weeks at 37ºC for bacterial colony enumeration and bacterial CFUs were determined using the formula:

Number of bacteria (CFU/mL) = number of colonies × 10 × dilution factor.

5.4.5 Experimental set-up for opsonophagocytic assay with isolated granulocytes and MNLs

Four systems (experimental sub-groups) were set up in a 24-well sterile plate (Merck KGsA, Darmstadt, Germany) per isolated human cell, as shown in **Table 5.1**. Cytokine production was measured in granulocytes or MNL cells when: unexposed as a negative control (sub-group 1), exposed to *M. tuberculosis* H37Rv (ATCC 26518) (sub-group 2), exposed to both H37Rv and mAb JG7 (sub-group 3), or to lipopolysaccharide (LPS) as a positive control (sub-group 4).

Table 5.1: Experimental set-up employed for each group of isolated granulocytes and mononuclear cells

	RPMI (µL)	Cells (µL)	Serum (µL)	JG7 (µL)	H37Rv (µL)	LPS (µL)	Sub-group description
Negative control (sub-group 1)	850	100	50	-	-	-	Cells only
Experimental Control (sub-group 2)	830	100	50	-	20	-	Cells, bacteria
Experiment (sub-group 3)	822	100	50	8	20	-	Cells, bacteria, mAb
Positive Control (sub-group 4)	840	100	50	-	-	10	Cells, LPS

RPMI (Roswell Park Memorial Institute-1640 medium); LPS (lipopolysaccharide); mAb (monoclonal antibody, JG7), H37Rv (virulent *M. tuberculosis* laboratory strain, ATCC 26518)

The final concentration of granulocytes and MNLs was 10^6 cells/mL and the final concentration of *M. tuberculosis* strain H37Rv was 10^7 CFU/mL. The mAb, JG7, was used at a final concentration of 25 µg/mL, and the positive control was stimulated with a final concentration of 10 µg/mL of LPS (Sigma-Aldrich GmbH, Munich, Germany).

5.4.6 Opsonophagocytic assay

The granulocytes and MNLs were incubated together with mAb JG7 for 20 minutes at 37ºC in a 5% carbon dioxide (CO_2) incubator (ESCO Technologies, Missouri, USA) prior to the addition of either *M. tuberculosis* strain H37Rv or LPS. Following the addition of bacteria and LPS, the cells were incubated for an additional 24 hours at 37ºC in a 5% CO_2 incubator. After the 24-hour incubation period, the well contents were transferred to 5 mL sterile tubes and centrifuged at 480 xg for 20 minutes at room temperature. The supernatants were drawn into clean eppendorf tubes

and frozen in a -80ºC freezer (Thermo Fisher Scientific, Massachusetts, USA) until use in the multiplex assay.

5.4.7 Multiplex suspension array assay procedure

The Milliplex MAP kit (Merck KGaA, Darmstadt, Germany) containing a standard human cytokine panel of seven cytokines including; IL-1β, IL-6, IL-8, IL-10, IL-12p70, IFN-γ and TNF-α, was used. All reagents were used at room temperature.

Wash buffer was used to pre-wash the 96-well assay plate. The wash buffer was decanted and 25 µL of each assay standard or control was dispensed into the allocated wells according to a pre-designed plate map. A volume of 25 µL of assay buffer was added into all the sample wells and 25 µL of matrix solution (RPMI-1640) was added to the background, standard and control wells. A 25 µL volume of sample (filtered supernatant) was added into designated wells. Pre-mixed cytokine beads were vortexed and 25 µL was added to each well.

The plate was sealed and incubated with gentle agitation on an orbital shaker (Thomas Scientific, Swedesboro, USA) for 2 hours at room temperature. After incubation, the plate was washed twice using an automated magnetic plate washer (Bio-Rad Laboratories Inc., California, USA). A volume of 25 µL of detection antibody was then added to each well and the plate was incubated on an orbital shaker with gentle agitation for 1 hour at room temperature. After incubation, 25 µL of Streptavidin-PE was added into each well and the plate was incubated for a final 30 minutes at room temperature on an orbital shaker. The plate was washed twice after incubation and 150 µL of sheath fluid (Bio-Rad Laboratories Inc., California, USA) was dispensed into each well. The plate was agitated for 2 minutes and read on the Luminex-200^{TM} suspension array system (Bio-Rad Laboratories Inc., California, USA).

Bio-Plex Manger software 6.0 was used for bead acquisition and analysis of median fluorescence intensity (MFI). A standard curve was generated by using the reference cytokine concentrations supplied by the manufacturer and the results are reported as pg/mL.

5.4.8 Multiplex immunoassay data analysis

Cytokine concentrations were obtained by measuring the MFI using a 5-parameter logistic or spline curve-fitting method. The Shapiro-Wilk test of normality was applied and since the data did not follow a normal distribution, descriptive statistics were non-parametric (median and inter-quartile range). The Kruskal Wallis test was used to measure the overall difference within the

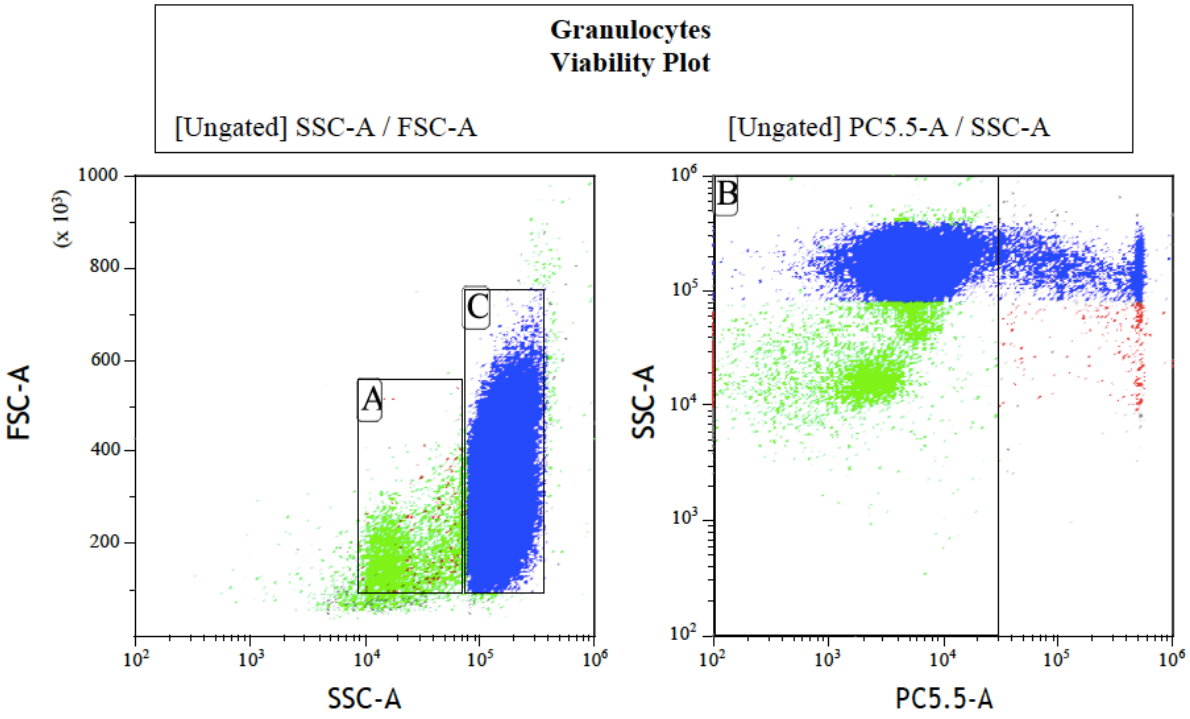
experimental sub-groups while the post-hoc Dunn's test was used to identify the differences between the experimental sub-groups by a pair-wise comparison.

5.5 Results

5.5.1 Human granulocytes

Flow cytometry

Flow cytometry was used to assess the cell number and percentage cell viability of the granulocyte cell population. **Figure 5.1** is a representative plot of the overall cell population (granulocytes plus other cell contaminants) alongside the gating strategy used in determining the granulocyte cell population. The viability plots for each of the four groups used are found in **Appendix D**, supplementary **Sheet 1.** Flow cytometry confirmed that the human granulocytes were viable for all four groups used, with viability ranging from 80% to 93%.



Gate	Number	%Total	%Gated	Cells/µL
All	95 757	100.00	100.00	958
A	4 410	4.61	4.61	44
C	**89 683**	**93.66**	**93.66**	**897**

Gate	Number	%Total	%Gated
All	95 757	100.00	100.00
B	91 330	95.38	95.38

Figure 5.1: Granulocyte cell population, cell number, and percentage viability *(Granulocytes: blue aggregates; gate C)*

Cytokine analysis from multiplex suspension array assay for human granulocytes

A panel of seven cytokines was used to investigate the interaction between granulocytes and either, a negative control (sub-group 1), *M. tuberculosis* H37Rv (ATCC 26518) (sub-group 2), both H37Rv and mAb JG7 (sub-group 3), or LPS as a positive control (sub-group 4). The raw data is available in **Appendix D**, supplementary **Sheet 2**. **Table 5.2** presents the median and interquartile range obtained for each of the seven cytokines investigated, per experimental sub-group.

Table 5.2: Descriptive statistics for experimental sub-groups per cytokine, expressed by human granulocytes

Cytokine	**Median** **Interquartile range**			
	Sub-group **1**	Sub-group **2**	Sub-group **3**	Sub-group **4**
IL-1β	303.70 182.49 – 651.27	1,062.70 656.64 – 1,151.32	1,183.35 781.87 – 1,428.92	1,010.48 691.62 – 1,415.47
IL-6	4,506.43 2,327.87 – 6,446.44	5,012.52 3,466.27 – 7,260.46	6,050.82 5,374.93 – 9,584.01	11,785.26 8,365.30 – 14,873.94
IL-8	13,320.62 11,664.83 – 16,687.22	13,506.36 11,826.43 – 17,924.58	17,367.61 15,304.67 – 30,722.14	21,753.28 17,985.53 – 27,779.50
IL-10	260.89 190.58 – 583.48	314.83 233.55 – 548.60	499.56 324.80 – 789.18	933.53 786.40 – 1,448.53
IL-12p70	38.76 9.06 – 49.34	32.56 26.35 – 49.34	26.35 9.06 – 38.76	70.09 37.85 – 114.32
IFN-γ	92.18 92.18 – 150.02	92.18 92.18 – 92.18	92.18 92.18 – 172.22	92.18 92.18 – 92.18
TNF-α	545.04 357.14 – 1,221.28	1,765.45 1,283.57 – 2,109.00	1,835.00 1,439.83 – 2,299.65	1,472.22 944.24 – 2,004.71

The data were further analysed by comparing cytokine production between experimental sub-groups (by a pair-wise comparison) for each cytokine investigated, with the granulocytes.

Table 5.3 gives the individual p-values obtained after comparing each experimental sub-group as well as the overall p-value obtained per cytokine, showing an overall significant difference among the sub-groups for four of the cytokines tested, namely IL-1β, IL-6, IL-10 and TNF-α, while IL-8 and IL-12p70 just missed significance.

Table 5.3: Pair-wise comparisons of cytokine production for experimental sub-groups with the human granulocytes

Cytokine	**Pair-wise group comparisons (p-values)**						
	1vs2	**1vs3**	**2vs3**	**1vs4**	**2vs4**	**3vs4**	**Overall p-value**
IL-1β	0.0165*	0.0018*	0.2198	0.0030*	0.2700	0.4365	0.0131*
IL-6	0.3746	0.0549	0.1004	0.0005*	0.0014*	0.0440*	0.0036*
IL-8	0.4365	0.0466*	0.0644	0.0102*	0.0154*	0.2612	0.0504
IL-10	0.3745	0.0611	0.1101	0.0006*	0.0017*	0.0440*	0.0046*
IL-12p70	0.4247	0.2158	0.1644	0.0288*	0.0438*	0.0036*	0.0533
IFN-γ	0.0689	0.5000	0.0689	0.0689	0.5000	0.0689	0.2208
TNF-α	0.0135*	0.0030*	0.2970	0.0492*	0.2878	0.1373	0.0371*

Table description: 1, 2, 3, and 4 (horizontally in bold) represent the experimental sub-groups. Therefore, 1vs2 represents a comparison between sub-groups 1 and 2, and so on. Each cytokine (vertically in bold) has a p-value that corresponds with a group comparison (values not in bold); p-values are presented with an asterisk [*] when the difference between the experimental sub-groups is statistically significant.

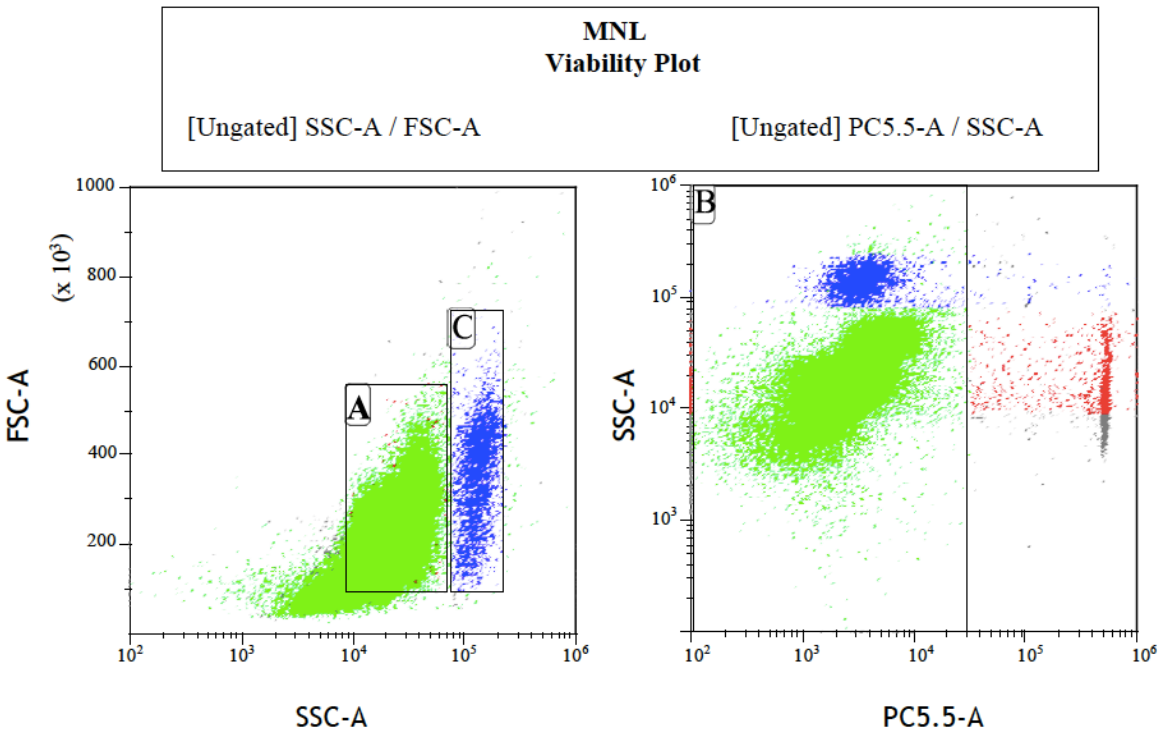
Sub-group 2 (experimental control containing cells and bacteria) had significantly higher levels of IL-1β and TNF-α than sub-group 1 (negative control containing cells only). Sub-group 3 (experiment containing cells, bacteria, and mAb JG7) followed a similar pattern, except it also had significantly higher levels of IL-8. As expected, all cytokine levels, except for IFN-γ, were significantly higher in sub-group 4 (positive control containing cells and LPS) when compared with sub-group 1. There was no significant difference between sub-groups 2 and 3 for any of the cytokines tested, although it should be noted that IL-8 and IFN-γ narrowly missed significance with p-values of 0.0644 and 0.0689 respectively. In this regard, IL-8 was higher in sub-group 3 and while the medians of IFN-γ were similar in the two sub-groups, the 75% percentile was much higher in sub-group 3. Sub-groups 2 and 3 were similar to sub-group 4 in terms of IL-1β, IFN-γ

and TNF-α, but had lower levels of IL-6, IL-10 and IL-12p70. In addition, sub-group 2 had a significantly lower level of IL-8 than sub-group 4.

5.5.2 Human MNL cells

Flow cytometry

Flow cytometry was used to assess the cell number and percentage cell viability of the MNLs. **Figure 5.2** is a representative plot of the overall cell population (MNLs plus other cell contaminants) alongside the gating strategy used in determining the overall MNL population. (See viability plots for each of the four groups in **Appendix D**, supplementary **Sheet 3).** MNL cell viability for all four groups was confirmed with viability ranging from 63% to 87%. The assay was also used to verify the different sub-populations within the MNL cell population.

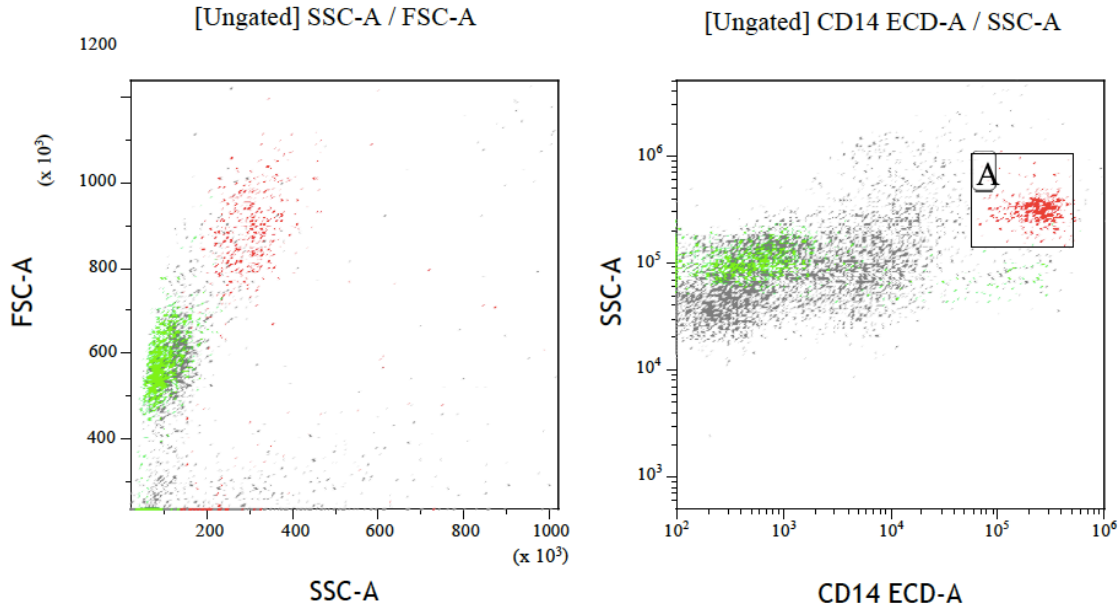


Gate	Number	%Total	%Gated	Cells/µL
All	123 681	100.00	100.00	1237
A	**107 604**	**87.00**	**87.00**	**1076**
C	3 956	3.20	3.20	40

Gate	Number	%Total	%Gated
All	123 681	100.00	100.00
B	121 373	98.13	98.13

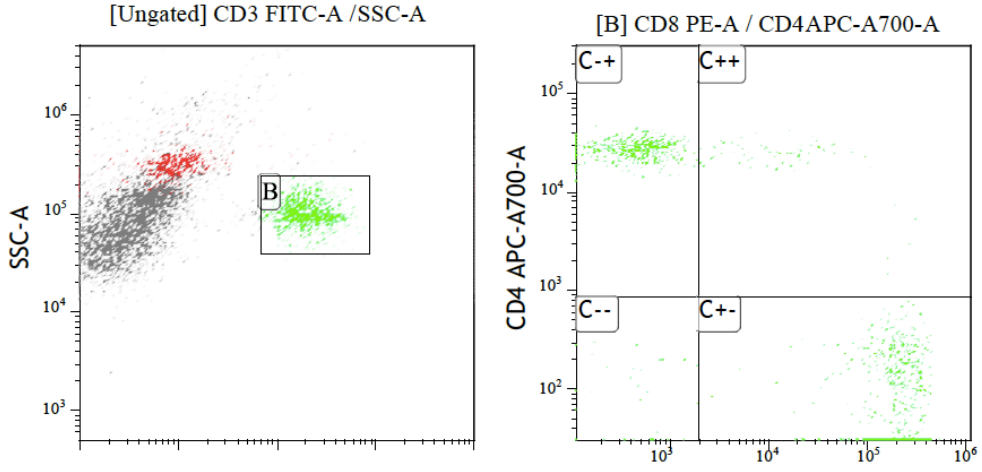
Figure 5.2: MNL cells, cell number, and percentage viability
(MNLs: green aggregates; gate A)

MNL - Plot of different cell populations



Gate	Number	%Total	%Gated
All	10000	100.00	100.00

Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
A	550	5.50	5.50



Gate	Number	%Total	%Gated
All	10000	100.00	100.00
B	1 221	12.21	12.21

Gate	Number	%Total	%Gated
All	1 221	12.21	100.00
C--	31	0.31	2.54
C-+	560	5.60	45.86
C+-	565	5.65	46.27
C++	65	0.65	5.32

Figure 5.3: Different MNL cell populations upon staining with different antibody markers, their cell numbers, and percentage viabilities

CD14 ECD antibody marker for the monocyte population (red aggregates; gate A); CD3 FITC antibody marker for the lymphocyte population (green aggregates; gate B): CD4 AF 700 and CD8 PE antibody markers for T cell populations (green aggregates; gates C--, C-+, C+-, C++)

Figure 5.3 shows histograms of cell numbers and percentage cell viabilities of the different sub-populations from the MNL cell population. **Appendix D**, supplementary **Sheet 4,** contains the histograms obtained for each of the four groups of MNL cells.

Flow cytometry was able to confirm the presence of different sub-populations within the MNL cell population, including, CD14+ monocytes, CD3+ lymphocytes, CD4+ and CD8+ T-cells.

Cytokine analysis from multiplex suspension array assay for human mononuclear cells

A panel of seven cytokines was used to investigate the interaction between granulocytes and either, a negative control (sub-group 1), *M. tuberculosis* H37Rv (ATCC 26518) (sub-group 2), both H37Rv and mAb JG7 (sub-group 3), or LPS as a positive control (sub-group 4). The raw data is available in **Appendix D**, supplementary **Sheet 5**. **Table 5.4** presents the median and interquartile range obtained for each of the seven cytokines investigated, per experimental sub-group.

Table 5.4: Descriptive statistics for experimental sub-groups per cytokine, expressed by human mononuclear cells

Cytokine	**Median** **Interquartile range**			
	Sub-group **1**	Sub-group **2**	Sub-group **3**	Sub-group **4**
IL-1β	187.09 127.54 – 415.87	7,617.34 7,549.45 – 7,923.89	8,003.80 7,734.92 – 8,055.93	3,851.27 2,859.31 – 6,086.69
IL-6	4,338.27 2,285.72 – 5,851.38	27,884.17 26,797.68 – 28,152.89	27,578.92 27,268.03 – 27,860.29	27,597.00 24,443.53 – 27,925.18
IL-8	8,609.76 7,870.15 – 9,126.76	10,925.53 8,762.35 – 11,891.01	12,034.47 10,682.17 – 12,945.86	10,553.83 9,103.86 – 12,893.86
IL-10	370.21 280.61 – 460.35	1,915.38 1,851.55 – 2,159.56	2,923.03 2,576.91 – 3,191.59	4,728.32 4,587.32 – 5,331.78
IL-12p70	32.56 17.71 – 44.05	126.79 90.69 – 200.25	54.08 32.56 – 67.28	58.92 46.48 – 100.23
IFN-γ	92.18 92.18 – 92.18	3,394.99 2,677.96 – 7,646.12	1,870.75 1,209.26 – 6,270.60	92.18 92.18 – 109.61
TNF-α	329.95 232.32 – 708.27	16,510.42 14,404.14 – 21,675.27	15,706.51 12,806.59 – 21,778.60	3,184.02 1,862.16 – 5,614.57

The data were further analysed by comparing the difference in cytokine production for each experimental sub-group (by a pair-wise comparison) for each cytokine investigated, with the MNLs. **Table 5.5** gives the individual p-values obtained after comparing each experimental sub-group as well as the overall p-value obtained per cytokine.

Table 5.5: Pair-wise comparisons of cytokine production for experimental sub-groups with the human mononuclear cells

Cytokine	**Pair-wise group comparisons (p-values)**						
	1vs2	**1vs3**	**2vs3**	**1vs4**	**2vs4**	**3vs4**	**Overall p-value**
IL-1β	0.0001*	< 0.0001*	0.1969	0.0440*	0.0165*	0.0014*	0.0001*
IL-6	< 0.0001*	0.0004*	0.2833	0.0015*	0.1720	0.3545	0.0004*
IL-8	0.0243*	0.0003*	0.0714	0.0088*	0.3447	0.1432	0.0062*
IL-10	0.0350*	0.0005*	0.0678	< 0.0001*	0.0005*	0.0350*	0.0001*
IL-12p70	< 0.0001*	0.0631	0.0040*	0.0073*	0.0406*	0.1808	0.0004*
IFN-γ	< 0.0001*	0.0004*	0.2018	0.3280	0.0001*	0.0017*	0.0001*
TNF-α	< 0.0001*	< 0.0001*	0.3746	0.0440*	0.0033*	0.0082*	0.0001*

Table description: 1, 2, 3, and 4 (rows, in bold) represent the experimental sub-groups. Therefore, 1vs2 represents a comparison between sub-groups 1 and 2, and so on. Each cytokine (columns, in bold) has a p-value that corresponds with a group comparison (values not in bold); p-values are presented with an asterisk [*] when the difference between the experimental sub-groups is statistically significant.

As shown in **Table 5.5**, an overall significant difference was observed among the sub-groups for all seven cytokines tested. All cytokine levels were significantly higher in sub-group 2 (experimental control containing cells and bacteria) when compared with sub-group 1 (negative control containing cells only). Sub-group 3 (experiment containing cells, bacteria, and mAb JG7) followed a similar pattern, except that it just missed significance with regards to IL-12p70. As expected, all cytokine levels, except for IFN-γ, were significantly higher in sub-group 4 (positive control containing cells and LPS) when compared with sub-group 1. There were no significant differences between sub-groups 2 and 3, except for IL-12p70 which was significantly higher in

sub-group 2. Higher levels of IL-1β, IFN-γ and TNF-α, and lower levels of IL-10, were observed in sub-groups 2 and 3 versus the positive control. In addition, sub-group 2 had a significantly higher level of IL-12p70 than sub-group 4.

5.6 Discussion

Cytokines are produced when there is an interaction between host cells and pathogens. There is a relationship between the cytokine response system, cell-mediated and antibody-mediated immunity and although the general consensus is that cytokines are essential to the host immune response, there is some controversy with regard to the exact roles of different cytokines in the immune response against *M. tuberculosis* infection. Cell-mediated responses are implicated in inflammation and destruction of infected cells, by driving the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-12, and IFN-γ while antibody-mediated responses are not only thought to initiate antibody response and macrophage/granulocyte activation, but also drive the production of anti-inflammatory cytokines such as IL-10 and IL-4 [Romero-Adrian *et al*., 2015; Domingo-Gonzalez *et al*., 2016]. An excessive pro-inflammatory immune response can result in a detrimental cytokine storm in which cytokines, such as IL-6, are important components [Goovaerts *et al*., 2013; Kiran *et al*., 2016]. In fact, a response involving the elevated production of the afore-mentioned cytokines is responsible for excessive lung inflammation and irreversible host tissue damage during *M. tuberculosis* infection [Zumla *et al*., 2015; Kolloli and Subbian, 2017]. The balance between pro- and anti-inflammatory responses is therefore critical since a shift towards either an excessive pro- or anti-inflammatory cytokine response may lead to the development of more aggressive disease or poor bacterial control, respectively [Lin *et al*., 2010; Coussens *et al*., 2014].

In the present study, two experimental sub-groups (sub-groups 2 and 3) were compared with two control sub-groups (sub-groups 1 and 4). The discussion is focused on comparisons between the experimental sub-groups, since they represent the interactions one would expect to occur in a biological system, upon *M. tuberculosis* infection (sub-group 2) and upon therapeutic intervention (sub-group 3). With regard to the granulocytes, levels of IL-8 were higher in sub-group 3 compared with sub-group 2 and, although the difference missed statistical significance ($p = 0.0644$), it is interesting to speculate whether the addition of the mAb could have increased neutrophil recruitment and function. IL-8 is a pro-inflammatory chemokine which is involved in

the recruitment of host immune cells, such as T lymphocytes and monocytes, but especially neutrophils, during *M. tuberculosis* infection [Alvarez-Jiménez *et al*., 2018]. Neutrophil numbers have been found to correlate with IL-8 levels in broncho-alveolar lavage fluid and with the expression of IL-8 mRNA in tuberculous lymph nodes [Krupa *et al*., 2015]. The increase in IL-8 production with this mAb intervention may be beneficial, since studies have demonstrated that IL-8 is able to stimulate neutrophil-dependent phagocytosis and killing of *M. tuberculosis* [Krupa *et al*., 2015; Romero-Adrian *et al*., 2015] and would be in keeping with the enhanced opsonophagocytosis reported in Chapter 4.

On the other hand, an excessive neutrophil response has been associated with TB disease severity, destruction of pulmonary tissue and poor outcomes [Mantovani *et al*., 2011; Lyadova, 2017]. When compared with non-destructive pulmonary infiltrates, a higher neutrophil: lymphocyte ratio has been found in tuberculous cavities [Barry *et al*., 2009]. In addition, upregulated secretion of neutrophil-derived matrix metalloproteinase-8 (MMP-8) has been demonstrated during *M. tuberculosis* infection and linked to matrix destruction, which leads to cavity formation [Ong *et al*., 2015]. It is therefore vital that exuberant neutrophil activation be countered by appropriate anti-inflammatory responses.

In this study, IL-8 production was also higher in sub-group 3 than in sub-group 2 with the MNL cells, although it just once again missed statistical significance ($p = 0.0714$). While contamination by neutrophils cannot be excluded, it is also possible that IL-8 had been produced by monocytes and macrophages infected with *M. tuberculosis,* since they, as well neutrophils and respiratory epithelial cells, have the ability to secrete IL-8.

Interestingly, and arguably the most important finding with MNL cells, is that IL-12p70 production was significantly lower in sub-group 3 than in sub-group 2. IL-12 belongs to the IL-6 super family of cytokines and the IL-12 family consists of a number of heterodimeric cytokines which lends it diverse and pleiotropic functions [Domingo-Gonzalez *et al*., 2016]. IL-12p70 is composed of the p35 and p40 subunits and is secreted primarily by macrophages, dendritic cells and B cells [Gee *et al*., 2009]. It is a pro-inflammatory cytokine which serves as a link between host innate and adaptive immunity [Romero-Adrian *et al*., 2015; Domingo-Gonzalez *et al*., 2016].

IL-12 plays a critical role in the immune response against *M. tuberculosis.* Phagocytic cells produce IL-12 upon phagocytosis of *M. tuberculosis*, which then, together with IL-18, induces

the differentiation of naïve $CD4^+$ T lymphocytes into IFN-γ-producing T-helper 1 effector lymphocytes [Ma *et al*., 2015]. The expression of IL-12 receptors is increased at the site of infection with *M. tuberculosis* and IL-12, together with IL-23, stimulates activation of antigen-specific CD4+ T-cells in the draining lymph nodes of *M. tuberculosis*-infected lungs. Animal experiments have shown that treatment of *M. tuberculosis*- infected mice with IL-12 lowers the bacterial burden while neutralization of IL-12 by mAbs, increases the bacterial burden and impairs granuloma integrity [Kumar *et al*., 2019]. Humans with deficiencies in IL-12Rβ1, IFNγR1, and IL-12p40, as found in Mendelian susceptibility to mycobacterial disease (MSMD), have a predisposition to infection with *M. tuberculosis,* nontuberculous mycobacterial infections, and are at risk of developing BCGosis after vaccination with the BCG vaccine [Cavalcanti *et al*., 2012; Domingo-Gonzalez *et al*., 2016].

In this study, the apparent downregulation of IL-12p70 could have been the result of the increased pro-inflammatory response driven by IL-8. This is supported by the observation of higher levels of IL-10 in sub-group 3 which, although just missing significance ($p = 0.0678$), is an expected response to exuberant inflammation. IL-10, formerly known as cytokine synthesis inhibitory factor, is widely viewed as the "master regulator of immunity to infection" [Couper *et al*., 2008]. It is produced by macrophages, dendritic cells, B cells, and various $CD4^+$ and $CD8^+$ T lymphocyte subsets and regulates T lymphocyte responses, inhibits MHC class II and costimulatory molecule expression on monocytes and macrophages, and limits the production of pro-inflammatory cytokines (including IL-12) and chemokines (including IL-8) [Couper *et al*., 2008; Cavalcanti *et al*., 2012].

An increase in IL-10 could be seen as detrimental to the *M. tuberculosis*-infected host since it could promote survival of mycobacteria is a number of ways: 1) deactivating macrophage function by downregulating IL-12 and TNF expression, thereby decreasing production of IFN-γ by T lymphocytes and reactive oxygen and nitrogen intermediates by macrophages; 2) inhibiting CD4+ T cell responses; 3) opposing antigen presentation by *M. tuberculosis*-infected cells by downregulating major histocompatibility complex molecules; and 4) delaying IL-12p40 dependent trafficking of dendritic cells carrying mycobacterial antigens to draining lymph nodes [Hossain and Norazmi, 2013]. However, IL-10 plays a key immunoregulatory role during various infections, including mycobacterial infections, where it limits the activation of both the innate and the adaptive immune cells with the aim of maintaining homeostasis [Ma *et al*., 2015]. IL-10 specifically counteracts excessive T-helper 1 responses (mostly characterised by IFN-γ and TNF-

α overproduction) which are associated with infection-related immunopathology [Couper *et al*., 2008]. In this regard, it is interesting to note that IFN-γ was indeed lower in sub-group 3 than in sub-group 2, although the difference was not statistically significant.

Many severe complications of infection are believed to be the result of excessive immune activation. For instance, mice lacking IL-27R signalling have been reported to control *M. tuberculosis* better than wild type mice but increased levels of TNF-α and IL-12p40 exacerbate inflammatory lung damage and cause animals to succumb more rapidly to disease [Cooper *et al*., 2007]. *In vitro* models have demonstrated that IFN-γ can cause lung damage through the enhanced release of lactate dehydrogenase from alveolar epithelial cells [Zhu *et al*., 2015]. In humans with active TB disease, elevated levels of TNF-α, IL-6, IL-8 and IL-12 in broncho-alveolar lavage fluid have been correlated with bronchial wall thickening, cavities, and fibrosis [Casarini *et al*., 1999; Woo *et al*., 2018].

In addition, increased tissue damage and mortality have been reported with several therapeutic and preventive strategies aimed at improving immune-mediated clearance of infection, including those utilizing adjuvant IL-12 therapy [Couper *et al*., 2008; Domingo-Gonzalez *et al*., 2016]. IL-12, due to its potency, has to be stringently controlled and IL-10 is one of its most important regulators [Domingo-Gonzalez *et al*., 2016].

While many studies have demonstrated the important role of many of the other cytokines assessed in this study, such as IL-1β [Domingo-Gonzalez *et al*., 2016; Thobakgale *et al*., 2017; Woo *et al*., 2018], IL-6 [Goovaerts *et al*., 2013; Kiran *et al*., 2016; Xiong *et al*., 2016], and TNF-α [Jayaraman *et al*., 2013; Thobakgale *et al*., 2017] for host defence against *M. tuberculosis* infection, this study did not demonstrate any significant modulation of expression of any of these cytokines by the mAb tested.

In conclusion, all the cytokines investigated in the present study, produce both beneficial and harmful effects in the host. For instance, the pro-inflammatory properties of a cytokine may cause severe, pro-inflammatory responses in the early stages of infection, which may be harmful to the host by promoting extensive tissue damage [Kiran *et al.*, 2016].

On the other hand, a delayed response may leave the host unprotected, thus, favouring unrestricted bacterial growth and dissemination at a later stage of infection [Zhang *et al*., 2011; Robinson *et al*., 2015; Kiran *et al*., 2016]. Therefore, a balance is needed in the host cytokine

response against *M. tuberculosis* infection. Human phagocytes contribute to the complicated and dynamic cytokine- and chemokine-mediated recruitment of additional inflammatory cells when early host defences fail to eradicate or significantly slow the growth of *M. tuberculosis,* thereby, serving as a link between the innate and adaptive host response [Orme, 2014; Kiran *et al*., 2016]. An intervention or HDT that is capable of restricting this excess cytokine recruitment in order to inhibit host tissue destruction is desirable.

Four different groups of human granulocytes and MNL cells were used in this study, and cells were isolated following a standard protocol. Cell viability ranged between 63% and 93% for both cell types, and opsonophagocytic assays were run immediately following cell isolation. Cell lines were not used in this study; firstly, since a series of preliminary studies (data not shown), demonstrated sub-optimal cytokine responses with the cell lines; and secondly, since the aim was to create a situation resembling the physiological setting to the greatest extent possible.

This study also had a few limitations including; the relatively small sample size with 64 experimental observations, the use of only one mAb concentration, and one *M. tuberculosis* strain, as well as investigating cytokine response at a single time point. Subsequently, a larger cohort will need to be investigated, in order to confirm the findings in this study.

5.7 Conclusion

A multiplex suspension array assay was used to assess the types of cytokines produced during a three-way human granulocyte/MNL cell - mAb - *M. tuberculosis* interaction, 24 hours after the process of mAb-enhanced phagocytosis or opsonophagocytosis of *M. tuberculosis* had taken place.

The human cell - mAb - bacteria interaction model used resulted in significant production of a number of important pro-inflammatory and anti-inflammatory cytokines at relatively high levels compared to the negative control.

The novel IgG1 mAb used in the study did not seem to have played a major role in modulating cytokine expression. However, a cautious interpretation of the results does suggest that the mAb increased the uptake of mycobacteria by neutrophils, possibly manifesting as a pro-inflammatory effect in these cells, while effecting a significant anti-inflammatory response in MNL cells. The most favourable outcome for a potential HDT should involve a mAb-enhanced treatment that

balances the cytokine response between pro- and anti-inflammatory cytokines, in order to better control *M. tuberculosis* infection, thereby limiting damage to infected and surrounding tissue. Additional studies need to therefore be conducted in order to fully assess the effect this novel mAb has on cytokine expression.

Ethical approval

All procedures performed were in accordance with the ethical standards of the institution's research committee (University of Pretoria Faculty of Health Sciences Research Ethics Committee, reference number-487/2018) and informed consent was obtained from each blood donor.

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5.8 References

Aberdein JD, Cole J, Bewley MA, Marriott HM, Dockrell DH (2013) Alveolar macrophages in pulmonary host defence the unrecognized role of apoptosis as a mechanism of intracellular bacterial killing. *Clinical and Experimental Immunology* **174**(2): 193–202.

Alvarez-Jiménez DV, Leyva-Paredes K, García-Martínez M, Vázquez-Flores L, García-Paredes GV, Campillo-Navarro M, Romo-Cruz I, Rosales-García HV, Castañeda-Casimiro J, González-Pozos S, Hernández MJ, Wong-Baeza C, García-Pérez EB, Ortiz-Navarrete V, Estrada-Parra S, Serafín-López J, Wong-Baeza I, Chacón-Salinas R, Estrada-García I (2018) Extracellular vesicles released from *Mycobacterium tuberculosis*-infected neutrophils promote macrophage autophagy and decrease intracellular mycobacterial survival. *Frontiers in Immunology* **9**: 272.

Cavalcanti VNY, Brelaz CAM, Neves K de-ALJ, Ferraz CJ, Pereira RAV (2012) Role of TNF-Alpha, IFN-Gamma, and IL-10 in the Development of Pulmonary Tuberculosis. *Pulmonary Medicine* doi: 10.1155/2012/745483.

Coussens AK, Martineau AR, Wilkinson RJ (2014) Anti-inflammatory and antimicrobial actions of vitamin D in combating TB/HIV. *Scientifica* doi: 10.1155/2014/903680.

D'Avila H, Roque NR, Cardoso RM, Castro-Faria-Neto HC, Melo RC, Bozza PT (2008) Neutrophils recruited to the site of *Mycobacterium bovis* BCG infection undergo apoptosis and modulate lipid body biogenesis and prostaglandin E production by macrophages. *Cell Microbiology* **10**(12): 2589–604.

Deretic V (2014) Autophagy in Tuberculosis. *Cold Spring Harbor Perspectives in Medicine* **4**: a018481.

Domingo-Gonzalez R, Prince O, Cooper A, Khader S (2016) Cytokines and Chemokines in *Mycobacterium tuberculosis* infection. *Microbiology Spectrum* doi: 10.1128/microbiolspec.TBTB2-0018-2016.

Gideon PH, Phuah J, Junecko AB, Mattila TJ (2019) Neutrophils express pro- and anti-inflammatory cytokines in granulomas from *Mycobacterium tuberculosis*-infected cynomolgus macaques. *Mucosal Immunology* **12**: 1370–1381.

Goletti D, Petruccioli E, Joosten AS, Ottenhoff HMT (2016) Tuberculosis biomarkers: from diagnosis to protection. *Infectious Disease Reports* **8**: 6568.

Goovaerts O, Jennes W, Massinga-Loembe M, Ceulemans A, Worodria W, Mayanja-Kizza H, Colebunders R, Kestens L, TB-IRIS Study Group (2013) LPS-binding protein and IL-6 mark paradoxical tuberculosis immune reconstitution inflammatory syndrome in HIV patients. *PLoS One* **8:** e81856.

Jacobs JA, Mongkolsapaya J, Screaton RG, McShane H, Wilkinson JR (2016) Antibodies and tuberculosis. *Tuberculosis* **101**: 102–113.

Jayaraman P, Sada-Ovalle I, Nishimura T, Anderson AC, Kuchroo VK, Remold HG, Behar SM (2013) IL-1beta promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *Journal of Immunology* **190**(8): 4196–4204.

Kiran D, Podell KB, Chambers M, Basaraba JR (2016) Host-directed therapy targeting the *Mycobacterium tuberculosis* granuloma: a review. *Seminars in Immunopathology* **38**: 167–183.

Kolloli A, Subbian S (2017) Host-Directed Therapeutic Strategies for Tuberculosis. *Frontiers in Medicine* **4**: 171.

Kozakiewicz L, Chen Y, Xu J, Wang Y, Dunussi-Joannopoulos K, Ou Q, Flynn JL, Porcelli SA, Jacobs WR Jr, Chan J (2013) B cells regulate neutrophilia during *Mycobacterium tuberculosis* infection and BCG vaccination by modulating the interleukin-17 response. *PLoS Pathogens* **9**(7): e1003472.

Krishnan N, Robertson DB, Thwaites G (2013) Pathways of IL-1b secretion by macrophages infected with clinical *Mycobacterium tuberculosis* strains. *Tuberculosis* **93**: 538–547.

Lin PL, Myers A, Smith L, Bigbee C, Bigbee M, Fuhrman C, Grieser H, Chiosea I, Voitenek NN, Capuano SV, Klein E, Flynn JL (2010) Tumor necrosis factor neutralization results in disseminated disease in acute and latent *Mycobacterium tuberculosis* infection with normal granuloma structure in a cynomolgus macaque model. *Arthritis and Rheumatism* **62**(2): 340–50.

Lyadova VI (2017) Neutrophils in Tuberculosis: Heterogeneity Shapes the Way? *Mediators of Inflammation* doi: https://doi.org/10.1155/2017/8619307.

Maglione PJ, Chan J (2009) How B cells shape the immune response against *Mycobacterium tuberculosis*. *European Journal of Immunology* **39**: 676–686.

Orme IM (2014) A new unifying theory of the pathogenesis of tuberculosis. *Tuberculosis* (*Edinb*) **94**(1): 8–14.

Ota OCM, Mendy FJ, Donkor S, Togun T, Daramy M, Gomez PM, Chegou NN, Sillah KA, Owolabi O, Kampmann B, Walzl G, Sutherland SJ (2014) Rapid diagnosis of tuberculosis using *ex vivo* host biomarkers in sputum. *European Respiratory Journal* **44**: 254–257.

Petruccioli E, Chiacchio T, Vanini V, Cuzzi G, Codecasa RL, Ferrarese M, Schinin V, Palmieri F, Ippolito G, Goletti D (2018) Effect of therapy on Quantiferon-Plus response in patients with active and latent tuberculosis infection. *Scientific Reports* **8**: 15626.

Robinson RT, Orme IM, Cooper AM (2015) The onset of adaptive immunity in the mouse model of tuberculosis and the factors that compromise its expression. *Immunology Reviews* **264**(1): 46–59.

Romero-Adrian BT, Leal-Montiel J, Fernández G, Valecillo A (2015) Role of cytokines and other factors involved in the *Mycobacterium tuberculosis* infection. *World Journal of Immunology* **5**(1): 16–50.

Sakhno LV, Shevela EY, Tikhonova MA, Nikonov SD, Ostanin AA, Chernykh ER (2015) Impairments of Antigen-Presenting Cells in Pulmonary Tuberculosis. *Journal of Immunology Research* doi: 10.1155/2015/793292.

Sei JC, Shey B, Schuman FR, Rikhi N, Muema K, Rodriguez DJ, Daum TL, Fourie PB, Fischer WG (2019) Opsonic Monoclonal Antibodies Enhance Phagocytic Killing Activity and Clearance of *Mycobacterium tuberculosis* from Blood in a Quantitative qPCR Mouse Model. *Heliyon* **5**(9): 1–10.

Thobakgale C, Naidoo K, McKinnon RL, Werner L, Samsunder N, Karim AS, Ndung'u T, Altfeld M, Naidoo K (2017) Interleukin 1-beta (IL-1β) production by innate cells following TLR stimulation correlates with TB recurrence in ART-treated HIV infected patients. *Journal of Acquired Immune Deficiency Syndrome* **74**(2): 213–220.

Walzl G, Ronacher K, Hanekom W, Scriba JT, Zumla A (2011) Immunological biomarkers of tuberculosis. *Nature Reviews Immunology* **11**: 343–354.

Weiss G, Schaible UE (2015) Macrophage defense mechanisms against intracellular bacteria. *Immunology Reviews* **264**(1): 182–203.

Woo M, Wood C, Kwon D, Park KH, Fejer G, Delorme V (2018) *Mycobacterium tuberculosis* Infection and Innate Responses in a New Model of Lung Alveolar Macrophages. *Frontiers in Immunology* **9**: 438.

Xiong W, Dong H, Wang J, Zou X, Wen Q, Luo W, Liu S, He J, Cai S, Ma L (2016) Analysis of Plasma Cytokine and Chemokine Profiles in Patients with and without Tuberculosis by Liquid Array-Based Multiplexed Immunoassays. *PLoS One* **11**(2): e0148885.

Zhang Q, Robin K, Liao D, Lambert G, Austin HR (2011) The Goldilocks principle and antibiotic resistance in bacteria. *Molecular Pharmaceutics* **8**(6): 2063–2068.

Zumla A, Rao M, Parida SK, Keshavjee S, Cassell G, Wallis R, Axelsson-Robertsson R, Doherty M, Andersson J, Maeurer M (2015) Inflammation and tuberculosis: host-directed therapies. *Journal of Internal Medicine* **277**(4): 373–87.

Chapter 6

6 Discussion and conclusions

6.1 General

Some progress has been made in the area of TB therapeutics, thereby increasing treatment success rates for both susceptible and multi-drug resistant forms of *M. tuberculosis.* Host-directed therapeutic (HDT) strategies have emerged in recent years as a potential avenue to facilitate this outcome. One such HDT, involving antibodies, demonstrates huge potential since it has several advantages over standard chemotherapy including: limited toxicity concerns, low risk of rejection (since it is not recognized as foreign to the body), and targeted action (directly modulating host cell functions). This targeted action helps in overcoming the development of drug resistance by the infecting *M. tuberculosis* strain and possibly affects several different mechanisms including constant fragment receptor (FcR)-mediated phagocytosis and autophagy (Zumla *et al.*, 2016; Kolloli and Subbian, 2017).

The importance of antibodies as a humoral immune response against TB cannot be overemphasized. Antibody-producing B-cells are higher in number in healthy controls compared to TB cases, and there is an increase in B-cell number at the end of successful anti-TB treatment (van Rensburg and Loxton, 2018). These B-cells also play a regulatory role, where they produce anti-inflammatory cytokines such as IL-10, thereby limiting inflammation upon *M. tuberculosis* clearance (van Rensburg and Loxton, 2018). Many schools of thought have come up with possible mechanisms employed by antibodies to neutralize *M. tuberculosis*; for instance, immune exclusion, wherein antibodies prevent the binding of *M. tuberculosis* adhesins to host cells by causing mycobacterial clumping. This may limit the dissemination of infection or prevent entry of *M. tuberculosis* into lung tissue (Jacobs *et al*., 2016). Another mechanism employed by these antibodies is surface binding to *M. tuberculosis* antigenic receptors. Investigating this targeted binding as the first step in a multi-step process towards the elimination of *M. tuberculosis* is important as antibody binding has been found to correlate with protection (Jacobs *et al*., 2016). Kawahara and colleagues also hypothesise that this receptor-binding action of antibodies, opsonizes bacteria, thereby enhancing their uptake by innate immune cells such as macrophages and enables these antibodies to serve as antigen presenters for adaptive components of the host's

immune system (Kawahara *et al*., 2019). FcR and particularly surface-binding monoclonal antibodies (mAbs) are thought to have neutralizing abilities and have even been shown to modulate cell-mediated immunity in experimental models (Jacobs *et al*., 2016; Focus/Editorial, 2018). These binding, neutralizing, and modulating activities of mAbs have been discussed in previous chapters.

The binding activities of two mAbs were assessed by different ELISAs. Monoclonal antibody binding activity was demonstrated for both susceptible and drug resistant *M. tuberculosis* at mAb concentrations as low as 0.156 µg/mL. The exact antigenic target(s) of the mAbs on the *M. tuberculosis* strains is yet unknown and future studies to determine these antigens will be helpful in vaccine research and development although preliminary findings hint at an epitope(s) on *M. tuberculosis* peptidoglycan being a possible target. Identifying the target(s) is important because, not only does it help narrow the focus with regards to which mAbs are most effective, but also implies that one could manipulate the binding sites of mAbs of interest. For example, the functional activity of mAbs could be maximized by modifying the glycan and FcR affinity of the mAbs and the attachment of an engineered Fc to a potently neutralizing antigen-binding fragment (Fab) may further improve its performance as an HDT (Focus/Editorial, 2018). Through this engineering process, Fan *et al*., (2010) have developed mAbs specific to the resuscitation promoting factor (Rpf) domain of *M. tuberculosis* strain H37Ra in mice and found it to be effective against the reactivation of latent H37Ra *in vivo* (Seidi and Jahanban-Esfahlan, 2013). The ability of mAbs to bind to surface antigens and increase a microorganism's sensitivity to drugs, via the modulation of gene expression, has also been shown in an experimental model of *Cryptococcus neoformans*, a fungal pathogen with a similar capsule as *M. tuberculosis* (McClelland *et al*., 2010, Cordero *et al*., 2013).

Monoclonal antibody-enhanced phagocytosis (opsonophagocytosis) was also investigated by *in vitro* opsonophagocytic (OP) assays. Opsonophagocytic killing activity (OPKA) was demonstrated by low bacterial CFUs relative to baseline (controls with no mAb) at mAb concentrations as low as 0.25 µg/mL. Comparatively, the lower JG7 concentrations (0.25 µg/mL and 0.5 µg/mL) did not seem as effective as the mid-range (1 µg/mL, 2.5 µg/mL, and 10 µg/mL) and higher concentrations (50 µg/mL and 100 µg/mL). The 10 µg/mL concentration performed best with the U-937 cell line while the 1 µg/mL concentration performed best with the HL-60 cell line. With regards to the bacterial dilutions assessed, OPKA was more effective at the lower bacterial dilution of 1:100 compared to the higher bacterial dilution of 1:1000 for both H37Ra

and cMtb in both human cell lines, with statistically significant differences in OPKA observed between dilutions 1:100 and 1:1000, for H37Ra with the HL-60 cell line and for cMtb with the U-937 cell line. Bacterial growth phase on the other hand, did not seem to affect OPKA, although, analytical statistics were not performed on the data set. This was a limitation of the study, as some co-factors investigated, such as cell line maturity, length of cell differentiation, and length of incubation, were not extensively analysed due to the relatively small sample size(s) used per factor. Therefore, more studies need to be conducted in order to confirm these findings.

Nevertheless, it has been successfully demonstrated that, a novel IgG1 mAb can be used as a potential therapeutic intervention against *M. tuberculosis*. This study is the first of its kind, especially in our setting, from the novel nature of the mAb, to the clinical susceptible TB strain and the variations in the *in vitro* assay used, as well as the factors investigated. The next step will involve: fully characterising the mAb in order to determine the exact antigenic target(s), assessing the functional capacity of this mAb to enhance the killing of drug resistant *M. tuberculosis* strains, and *in vivo* assessments in animal models alongside the use of standard antimicrobial chemotherapeutic agents, in order to assess the clearance ability of the mAb during and after chemotherapy. The expectation is that a potential intervention like this should be able to perform across the spectrum of drug resistance and, as is the case with binding activity, OPKA should be similar between susceptible and resistant *M. tuberculosis* strains. In addition, *M. tuberculosis* clearance should also increase with the use of standard anti-TB regimens, since the mAb intervention is expected to act in synergy with chemotherapeutic agents.

Finally, the cytokine response to opsonophagocytosis was assessed by multiplex suspension array assays. Statistically significant increases in cytokine production were observed when the cells were exposed either to; bacteria, bacteria and mAb, or LPS. This observation is consistent with findings by Woo and colleagues, who demonstrate that live *M. tuberculosis* induces a higher production of pro-inflammatory cytokines in Max Planck Institute cells compared to heat-killed bacteria, due to its intracellular replication (Woo *et al*., 2018). Comparing the level of cytokine production between the bacteria exposed and the bacteria and mAb exposed groups, a large but non-significant increase in IL-8 expression was observed with the granulocytes exposed to the mAb. In contrast, the mAb-exposed MNL cells expressed significantly lower levels of IL-12p70, together with a large but non-significant increase in IL-10 expression. The mAb JG7 did not seem to have played a major role in modulating cytokine expression, although it probably increased *M. tuberculosis* phagocytosis in the granulocytes, as shown by the increased pro-

inflammatory effect in these cells. This, coupled with the significant anti-inflammatory response demonstrated in MNL cells, leads to the conclusion that this novel IgG1 mAb plays a role in the cytokine response, although, the experimental cohort was limited, and a wider cohort will help in assessing the validity of these observations. Nevertheless, it has been demonstrated that cytokines are expressed upon *M. tuberculosis* interaction with human phagocytes, and gone a step further, to show that a three-way interaction involving an additional mAb component, also produces a number of immunologically important cytokines whose individual expressions are either augmented or suppressed with mAb intervention. This kind of investigation has never been conducted in our setting and as is the case with the opsonophagocytic assays, the next step will involve measuring the cytokine response, specifically upon mAb intervention, in a biological system (animal experiments).

In conclusion, the idea of using mAbs as future therapeutic agents in TB is a profound concept that warrants greater consideration. Phagocytosis is improved by opsonization, as has been specifically demonstrated for *M. tuberculosis* by Bangani *et al*., (2016) but finding mAbs that are capable of opsonizing different *M. tuberculosis* strains at different stages of infection is a huge challenge due to their specific nature. This step has successfully been achieved by demonstrating *in vitro,* that this novel mAb binds to various *M. tuberculosis* strains at both their mid-logarithmic and stationary phases of growth. There has also been evidence to demonstrate that the mAb enhances *M. tuberculosis* phagocytosis in human macrophage and granulocyte cell lines. Finally, this novel IgG1 mAb may not only serve as a good therapeutic intervention against *M. tuberculosis*, but also have a modulatory effect on both pro- and anti-inflammatory cytokine responses. The performance of this novel mAb at this stage, leads us to believe that its additive effect when used in combination with chemotherapy will be phenomenal hence, the conclusion that mAbs are the future for HDTs against TB.

Given current advances in antibody research, the use of mAbs for therapeutic vaccination looks promising. There might be a shift from using mAbs as adjunctive therapy, to employing them as booster treatments, specifically for preventive measures. Also, considering the complementarity of mAbs to different antigens (as a result of numerous binding sites on the mAb), this approach might help eliminate the need for disease-specific treatment regimens in favour of more broad-spectrum applications.

6.2 References

Cordero RJB, Pontes B, Frases S, Nakouzi AS, Nimrichter L, Marcio L, Viana NB, Casadevall A, Rodrigues ML (2013) Antibody binding to *Cryptococcus neoformans* impairs budding by altering capsular mechanical properties. *Immunology* **190**: 317–23.

Fan A, Jian W, Shi C, Ma Y, Wang L, Peng D, Bai Y, An Q, Hao X, Xu Z (2010) Production and characterization of monoclonal antibody against *Mycobacterium tuberculosis* RpfB domain. *Hybridoma (Larchmt)* **29**(4): 327–32.

Focus/EDITORIAL (2018) Old foes and new enemies. *Nature Immunology* **19**: 1147.

Jacobs JA, Mongkolsapaya J, Screaton RG, McShane H, Wilkinson JR (2016) Antibodies and tuberculosis. *Tuberculosis* **101**: 102–113.

Kawahara JY, Irvine EB, Alter G (2019) A Case for Antibodies as Mechanistic Correlates of Immunity in Tuberculosis. *Frontiers in Immunology* **10**: 996.

Kolloli A, Subbian S (2017) Host-Directed Therapeutic Strategies for Tuberculosis. *Frontiers in Medicine* **4**: 171.

McClelland EE, Nicola AM, Prados-Rosales R, Casadevall A (2010) Ab binding alters gene expression in *Cryptococcus neoformans* and directly modulates fungal metabolism. *Clinical Investigation* **120**: 1355–61.

Seidi K, Jahanban-Esfahlan R (2013) A novel approach to eradicate latent TB: Based on resuscitation promoting factors. *Journal of Medical Hypotheses and Ideas* **7**: 69–74.

van Rensburg CI, Loxton GA (2018) Killer (FASL regulatory) B cells are present during latent TB and are induced by BCG stimulation in participants with and without latent tuberculosis. *Tuberculosis* **108**: 114–117.

Woo M, Wood C, Kwon D, Park KH, Fejer G, Delorme V (2018) *Mycobacterium tuberculosis* Infection and Innate Responses in a New Model of Lung Alveolar Macrophages. *Frontiers in Immunology* **9**: 438.

Zumla A, Rao M, Wallis SR, Kaufmann HES, Rustomjee R, Mwaba P, Vilaplana C, Yeboah-Manu D, Chakaya J, Ippolito G, Azhar E, Hoelscher M, Maeurer M, Host-Directed Therapies Network consortium (2016) Host-directed therapies for infectious diseases: current status, recent progress, and future prospects. *Lancet Infectious Diseases* **16:** e47–63.

Appendix A: Reagents, stock concentrations, buffers and media used

Reagent combinations

Complete growth medium (CGM):

- 1 L of Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma-Aldrich GmbH, Munich, Germany)
- 11 mL of 200 mM L-glutamine (Sigma-Aldrich GmbH, Munich, Germany)
- 112 mL of foetal bovine serum - FBS (Lasec, Cape Town, SA), (Biocom Diagnostics, Centurion, SA)

Differentiation medium, U-937:

- 1.25 µL (50 ng/mL) of Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich GmbH, Munich, Germany)
- 25 mL of CGM

Differentiation medium, HL-60:

- 100 mL of RPMI-1640
- 1.1 mL L-glutamine
- 20% FBS
- 1.25% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich GmbH, Munich, Germany)

OPKA medium:

- 25 mL of Dulbecco's Modified Eagle Medium Nutrient Mixture 12 (DMEM F-12) (Sigma-Aldrich GmbH, Munich, Germany)
- 510 µL of 10 mM hydroxyethyl piperazine-ethane-sulfonic acid (HEPES) (Sigma-Aldrich GmbH, Munich, Germany)

Bovine serum albumin (BSA):

- 30% BSA (Sigma-Aldrich GmbH, Munich, Germany)
- 0.1% BSA: 133.3 µL of 30% BSA in 40 mL of tissue grade water (Biocom Diagnostics, Centurion, SA)

Stock concentrations (1 mg/mL)

Human C1q complement: 1 mg (Biocom Diagnostics, Centurion, SA) in 1 mL of deionized water and further dilute at a ratio of 1:5 or 1:10 in OP assay medium

JG7: 1.24 mg/mL, 1.30 mg/mL, or 3.04 mg/mL of JG7 (Longhorn Vaccines and Diagnostics, Bethesda, USA) in different volumes of OP assay medium for various concentrations as shown below:

JG7 Lot XXX → 1.24 mg/mL

Dilution factor	Volumes	JG7 µg/mL
1:12.4 →	56.45uL of mAb in 643.55uL of Assay Media →	100.00
1:24.8 →	34.27uL of mAb in 815.73uL of Assay Media →	50.00
1:49.6 →	14.1uL of mAb in 685.9uL of Assay Media →	25.00
1:124 →	5.65uL of mAb in 694.36uL of Assay Media →	10.00
1:20 from 50ug/ml →	35uL of stock in 665uL of Assay Media →	2.50
1:50 from 50ug/ml →	14uL of stock in 686uL of Assay Media →	1.00
1:100 from 50ug/ml →	7uL of stock in 693uL of Assay Media →	0.50
1:200 from 50ug/ml →	3.5uL of stock in 696.5uL of Assay Media →	0.25

JG7 Lot XXX → 1.30 mg/mL

Dilution factor	Volumes	JG7 µg/mL
1:13 →	53.85uL of mAb in 646.15uL of Assay Media →	100.00
1:26 →	32.69uL of mAb in 817.31uL of Assay Media →	50.00
1:52 →	13.46uL of mAb in 686.54uL of Assay Media →	25.00
1:130 →	5.39uL of mAb in 694.61uL of Assay Media →	10.00
1:20 from 50ug/ml →	35uL of stock in 665uL of Assay Media →	2.50
1:50 from 50ug/ml →	14uL of stock in 686uL of Assay Media →	1.00
1:100 from 50ug/ml →	7uL of stock in 693uL of Assay Media →	0.50
1:200 from 50ug/ml →	3.5uL of stock in 696.5uL of Assay Media →	0.25

JG7 Lot XXX → 3.04 mg/mL

Dilution factor	Volumes	JG7 µg/mL
1:121.6 →	4.53uL of mAb in 545.47uL of Assay Media →	25.00

Buffers

- 1L of Phosphate buffered saline (PBS), pH - 7.4: 1 tablet of PBS (Sigma-Aldrich GmbH, Munich, Germany) in 1L of distilled water and autoclave for 15 minutes at 121ºC

- 1L of Phosphate buffered saline plus tween 20 (PBS-T), pH - 7.2: 1 sachet of PBS-T (Sigma-Aldrich GmbH, Munich, Germany) in 1L of distilled water and filter using IL filter system (Thermo Fisher Scientific, Massachusetts, USA)
- 3% gelatine (Merck KGaA, Darmstadt, Germany) in PBS, pH - 7.4: 6 g of gelatine powder in 200 mL of distilled water and add 1.846 g of PBS

Media

900 mL of Middlebrook 7H9 broth:

- 4.7 g of Middlebrook 7H9 powder (Becton Dickinson, New Jersey, USA)
- 2 mL of glycerol (Thermo Fisher Scientific, Massachusetts, USA)

900 mL of Middlebrook 7H10 agar:

- 19 g of Middlebrook 7H10 powder (Becton Dickinson, New Jersey, USA)
- 5 mL of glycerol
- 200 mL of oleic acid, agarose, dextrose catalase (OADC) and polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin (PANTA) mixture (Becton Dickinson, New Jersey, USA) mixture

900 mL of Middlebrook 7H11 agar:

- 19 g of Middlebrook 7H11 powder (Becton Dickinson, New Jersey, USA)
- 5 mL of glycerol
- 200 mL of OADC-PANTA mixture (Becton Dickinson, New Jersey, USA)

List of Suppliers

- Becton Dickinson, New Jersey, United States of America.
- Thermo Fisher Scientific, Massachusetts, United States of America.
- Lasec, Cape Town, South Africa.
- Biocom Diagnostics, Centurion, South Africa.
- Sigma-Aldrich GmbH, Munich, Germany.
- Longhorn Vaccines and Diagnostics, Bethesda, United States of America.
- Merck KGaA, Darmstadt, Germany.
- Beckman Coulter, California, United States of America.

Appendix B: Experimental procedures

Reconstituting and growing H37Ra from initial stock

- To four mycobacterial growth incubation tubes (MGITs), add **800 µL** of the oleic acid, albumin, dextrose, catalase – polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin (OADC-PANTA) mixture into each tube.
- Take out the swab containing powdered ATCC H37Ra and insert into the first MGIT for the bacteria to disperse into the medium.
- From the first "neat" tube, carry out consecutive 1:10 serial dilutions into subsequent tubes.
- Seal the first two tubes in parafilm and store at -20ºC.
- Incubate the other tubes in the BACTEC system for 2-3 weeks.

H37Ra colony enumeration on solid media

- Take out the MGITs after they flake positive and record the bacterial growth unit (GU) (determined by the BACTEC system).
- Serially dilute the bacteria in phosphate buffered saline (PBS).
- Plate each serial dilution on Middlebrook 7H10 or 7H11 agars, and its corresponding counterpart in MGIT; incubate at 37ºC for 6-10 weeks and 2-3weeks respectively.
- Continue the process of diluting a positive tube from MGIT and sub-culturing on both solid and liquid media until the bacteria can be enumerated on agar.
- The GU from the BACTEC system can then be compared with the bacterial counts on agar to assess the number of colony-forming units (CFUs) on agar, that correspond to a particular GU.

Sub-culturing H37Rv in liquid medium

- Aliquot Middlebrook 7H9 broth containing 0.05% Tween-80, 0.2% glycerol, and 10% OADC into sterile Schott bottles.
- Add approximately 10^5 CFU/mL of the bacteria into the broth.
- Incubate the cultures at 37°C in a shaking incubator until an OD of 0.6 - 1.0 is reached, at 540 nm (measure using a spectrophotometer).
- Centrifuge the bacterial culture at 480 xg for 10 minutes at room temperature, discard the supernatant, and wash the pellet with **50 mL** of PBS (pH 7.4).

- Add approximately **5 mL** of glass beads (3 mm) to the pellet and vortex to disperse the clumps.
- Re-suspend the bacteria in sterile PBS and adjust the bacterial suspension to an OD of 1.2 at 540 nm (approximately 10^8 CFU/mL).

Protocol for cell culture of human monocyte cell line U-937

- To a **1L** bottle of Roswell Park Memorial Institute (RPMI)-1640, add **11 mL** of L-glutamine and **112 mL** of heat-inactivated foetal bovine serum (FBS).
- Filter the resulting mixture using a IL filter system.
  - **NB:** This culture passing medium becomes complete growth medium (CGM) and can be stored for up to 2 weeks at 4ºC, after the addition of L-glutamine.
- Heat the CGM in a water bath at 37ºC for 30 minutes each time the cells need to be passaged.
- Take T-flasks (start with T-25 flasks, then T-75 flasks, and then T-125 flasks) from the incubator and gently transfer its contents into sterile conical tubes.
- Centrifuge the tubes at 500 xg for 5 minutes at 25ºC.
- Discard the supernatant and re-suspend the pellet in **40 - 80 mL** of pre-heated CGM.
- Vortex and remove **100 µL** of the mixture (use serological pipette); and add to **900 µL** of trypan blue (1:10 dilution).
  - **NB:** This is for performing a viable cell count using a haemocytometer. Count the cells in a 1 mm^3 square.
- Live and dead cells should be counted and recorded separately (dead cells take up the dye and become dark blue while live cells are colourless).
- Record viability as: 1-(dead cells/live cells) x 100.
- Record the number of cells/mL as: live cells/number of squares counted x trypan blue dilution x 10^4.
- Dilute the cells to the appropriate target concentration of **$1.5x10^5$ cells/mL,** in CGM.
- Pour into the appropriate-sized T-flask, label, and incubate in a 5% CO_2 incubator for 3 - 4 days.

For differentiation,

- To a **1L** bottle of RPMI-1640, add **11 mL** of L-glutamine and **112 mL** of heat-inactivated FBS.

- Add **56.15 µL** of Phorbol 12-myristate 13-acetate (PMA) at a final concentration of **50 ng/mL** (1:20,000 of PMA @ 1 mg/mL).
- OR, if differentiating the cells directly on a 96-well plate, add **2.5 µL** of PMA @ 1 mg/mL to **50 mL** of the CGM.
  - **NB;** This becomes the differentiation medium.
- After performing cell counts as described above, dilute the cells to the target concentration of **$1.5x10^5$ cells/mL** or if using a 96-well plate, **$0.5x10^6$ cells/mL/well.**
- Pour into the appropriate-sized T-flask, label, and incubate in a 5% CO_2 incubator for 3 - 4 days.

Protocol for cell culture of human granulocyte cell line HL-60

- To a **1L** bottle of RPMI-1640, add **11 mL** of L-glutamine and **112 mL** of heat-inactivated FBS and filter the resulting mixture using a IL filter system.
  - **NB:** This culture passing medium becomes CGM and can be stored for up to 2 weeks at 4ºC, after the addition of L-glutamine.
- Heat the CGM in a water bath at 37ºC for 30 minutes each time the cells need to be passaged.
- Take T-flasks (start with T-25 flasks, then T-75 flasks, and then T-125 flasks) from the incubator and gently transfer its contents into sterile conical tubes.
- Centrifuge the tubes at 500 xg for 5 minutes at 25ºC.
- Discard the supernatant and re-suspend the pellet in **40 - 80 mL** of pre-heated CGM.
- Vortex and remove **100 µL** of the mixture (use serological pipette); and add to **900 µL** of trypan blue (1:10 dilution).
  - **NB:** This is for performing a viable cell count using a haemocytometer. Count the cells in a 1 mm^3 square.
- Live and dead cells should be counted and recorded separately (dead cells take up the dye and become dark blue while live cells are colourless).
- Record viability as: 1-(dead cells/live cells) x 100.
- Record the number of cells/mL as: live cells/number of squares counted x trypan blue dilution x 10^4.
- Dilute the cells to the appropriate target concentration of **$1.5x10^5$ cells/mL,** in CGM.
- Pour into the appropriate-sized T-flask, label, and incubate in a 5% CO_2 incubator for 3 - 4 days.

For differentiation,

- To a **1L** bottle of RPMI-1640, add **11 mL** of L-glutamine, **240 mL** of heat-inactivated FBS, and **15.2 mL** of dimethyl sulfoxide (DMSO).
  - **NB;** This becomes the differentiation medium
- After performing cell counts as described above, dilute the cells to the target concentration of **$2x10^5$ cells/mL** in differentiation medium
- Pour into the appropriate-sized T-flask, label, and incubate in a 5% CO_2 incubator for 5 - 6 days.

Protocol for *in vitro* opsonophagocytic assay using U-937 cells

Preparation of M. tuberculosis strains for the OP assay

- Grow pure cultures of *M. tuberculosis* (H37Ra or clinical isolate) in MGIT 7H9 broth at 37ºC.
- Incubate till mid-logarithmic phase is reached at a range of approximately **1600 GU - 3000 GU** ($1x10^6$ CFU/mL - $1x10^8$ CFU/mL).
- Take the incubation tube out and vortex it vigorously.
- Take **500 µL** of bacteria from the tube and serially dilute it in 4 tubes, each containing **4.5 mL** of 7H9 broth.
- Use the last two bacterial dilutions (1:100 and 1:1000 or 1:1000 and 1:10000) for each assay.
- *In vitro OP assay*
- To a **500 mL** bottle of Dulbecco's Modified Eagle Medium Nutrient Mixture 12 (DMEM/F12), add **10.2 mL** of 1M hydroxyethyl piperazine-ethane-sulfonic acid (HEPES).
  - **NB**: This becomes the assay medium.
- Add **0.1 mL** of 30% bovine serum albumin (BSA) to **30 mL** of tissue culture grade or sterile water.
- Take out **2** sterile, 96-well round bottom plates and label them "dilution plate*"* and "incubation plate*"*.
- Aliquot **200 mL** of the assay medium and pre-heat at 37ºC, for re-suspending the U-937 cells.
- Aliquot **25 mL** of the assay medium for diluting monoclonal antibody (mAb) JG7.
- Prepare different dilutions (concentrations) of JG7 and aliquot **60 µL** of each dilution into each well of the dilution plate.

- Remove previously sub-cultured H37Ra or clinical susceptible TB at mid-logarithmic phase from the BACTEC system and record the GU.
- Remove **50 µL** of the bacterial suspension and add to **4950 µL** of 7H9 broth (1:100).
- Add **10 µL** of the diluted bacterial suspension to each well of the plate.
- Transfer differentiated U-937 cells from the T-flasks into conical tubes.
- Wash the T-flasks by adding **10 mL** of sterile PBS twice.
- Add **7 mL** of trypsin-versene (so that the entire flask surface is covered) and incubate at 37ºC for 12 minutes.
- After incubation, gently scrape the cells off the flask surface with a cell scraper and add **7 mL** of warm assay medium.
- Gently pipette up and down across the flask surface, to take off all the cells, and add the contents into the conical tubes containing the U-937 cells.
- Centrifuge the tubes at 500 xg for 5 minutes at 25ºC.
- Discard the supernatant and re-suspend the pellet in **50 mL** of warm assay medium (total volume).
- Extract **100 µL** of U-937 cells from the prepared 50 mL suspension and add to **900 µL** of trypan blue.
- Count the cells using a haemocytometer and record the number of cells per mL.
- Centrifuge the cells (**50 mL**) again at 500 xg for 5 minutes at 25ºC.
- Discard the supernatant and dilute the cells to a concentration of **$5x10^7$ cells/mL** using warm assay medium.
- If cells were differentiated in a 96-well plate, remove the CGM from the top of the cells and add **50 µL** of warm assay medium per well (this becomes the incubation plate).
- Transfer **40 µL** of JG7 from the dilution plate, to the corresponding wells on the incubation plate.
- Transfer **10 µL** of the bacteria to each well, seal the plate and incubate in a shaking incubator at 300 rpm for 4 hours at 37ºC.
- After incubation, place the plate on ice and pour the previously prepared BSA solution into a reservoir (hold on ice).
- Discard **90 µL** of solution from each well of the plate.
- Add **190 µL** of BSA to each well, mix gently, and incubate the plate on ice for 30 minutes.

- Transfer **100 µL** from each well onto the corresponding pre-labelled 7H10 or 7H11 agar plate.
- Incubate the agar plates at 37ºC for 6 - 10 weeks, and count the colonies using an automated colony counter.

Protocol for *in vitro* opsonophagocytic assay using HL-60 cells

Preparation of M. tuberculosis strains for the OP assay

Use the same bacterial preparation procedure as that used for the *in vitro* OP assay with the HL-60 cells.

In vitro OP assay

- To a **500 mL** bottle of DMEM/F12, add **10.2 mL** of 1M HEPES (this becomes the assay medium).
- Add **0.1 mL** of 30% BSA to **30 mL** of tissue culture grade or sterile water.
- Take out **3** sterile, 96-well round bottom plates and label them "dilution plate", "incubation plate" and "final dilution plate".
- Aliquot **100 mL** of the assay medium and pre-heat at 37ºC, for re-suspending the HL-60 cells.
- Aliquot **5 mL** of the assay medium for diluting complement component C1q, and place on ice.
- Aliquot **50 mL** of the assay medium for diluting mAb JG7.
- Prepare different dilutions (concentrations) of JG7 and aliquot **60 µL** of each dilution into each well of the dilution plate.
- Remove previously sub-cultured H37Ra or clinical susceptible TB at mid-logarithmic phase from the BACTEC system and record the GU.
- Remove **50 µL** of the bacterial suspension and add to **4950 µL** of 7H9 broth (1:100).
- Add **10 µL** of the diluted bacterial suspension to each well of the plate.
- Place complement component C1q on ice.
- Transfer the differentiated HL-60 cells from the T-flasks into conical tubes and centrifuge the tubes at 500 xg for 5 minutes at 25ºC.

- Discard the supernatant and re-suspend the pellet in **50 mL** of warm assay medium (total volume).
- Extract **100 µL** of HL-60 cells from the prepared 50 mL suspension and add to **900 µL** of trypan blue.
- Count the cells using a haemocytometer and record the number of cells per mL.
- Centrifuge the cells (**50 mL**) again at 500 xg for 5 minutes at 25ºC.
- Discard the supernatant and dilute the cells to a concentration of **$5x10^7$ cells/mL** using warm assay medium.
- Transfer **40 µL** of JG7 from the dilution plate, to the corresponding wells on the incubation plate.
- Dilute the C1q in previously assigned assay medium on ice, vortex once, and transfer into a reservoir held on ice.
- Transfer **10 µL** of the C1q mixture into each well of the incubation plate.
- Transfer **10 µL** of the bacteria to each well, seal the plate and incubate in a shaking incubator at 300 rpm for 4 hours at 37ºC.
- After incubation, place the plate on ice and pour the previously prepared BSA solution into a reservoir (hold on ice).
- Transfer **190 µL** of previously prepared BSA into each well of the final dilution plate on ice.
- Mix the solution in each well of the incubation plate and transfer **10 µL** from each well into the corresponding wells of the final dilution plate containing BSA.
- Incubate the plate on ice for 30 minutes.
- Transfer **100 µL** from each well onto the corresponding pre-labelled 7H10 or 7H11 agar plate.
- Incubate the agar plates at 37ºC for 6 - 10 weeks, and count the colonies using an automated colony counter.

Protocol for human granulocyte cell isolation

- Aliquot **15 mL** of Histopaque-1077 into six 50 mL falcon tubes.
- Slowly layer heparinised venous blood (5 units of preservative-free heparin per mL) onto the Histopaque-1077 and make up to the **50 mL** mark.
- Centrifuge the tubes at 1800 rpm for 25 minutes at 121ºC.

- Separate the granulocyte cell layer from the mononuclear cell layer by placing each into different 50 mL falcon tubes.
- Make a 3% gelatine solution with PBS by adding **1.846 g** of PBS powder to **6 g** of gelatine powder and dilute the mixture in **200 mL** of distilled water.
- Heat the solution for 2 minutes in a microwave and maintain the temperature at 56ºC in a water bath.
- Add the gelatine mixture to each falcon tube containing approximately **20 mL** of the separated granulocyte cells and make up to the **50 mL** mark.
- Mix the solution and incubate at 37ºC for 15 minutes.
- Pool the supernatants and centrifuge at 1200 rpm for 10 minutes at 25ºC.
- Discard the supernatants, vortex the pellets and fill the tubes with sterile ammonium chloride (make up to **50 mL** mark).
- Place the tubes on ice for 10 minutes.
- Centrifuge the tubes at 1200 rpm for 10 minutes at 25ºC.
- Discard the supernatants, vortex the pellets, and pool the cells into one falcon tube.
- Add PBS up to the **50 mL** mark and centrifuge at 1200 rpm for 10 minutes at 25ºC.
- Discard the supernatant, vortex the pellet, and re-suspend the cells in **3 - 5 mL** of RPMI-1640.
- Count the cells microscopically, using a haemocytometer (1:10 ratio of cell suspension: gentian violet solution).
- Based on the cell count, re-suspend the cells in a volume of RPMI-1640 that gives a final concentration of **10^7 cells/mL.**

Protocol for human mononuclear (MNL) cell isolation

- Prepare a PBS - EGTA mixture at a concentration of **300 µM**, at 25ºC.
- Add the mixture to each falcon tube containing **15 mL** of the separated MNLs and make up to the **50 mL** mark.
- Centrifuge the tubes at 1200 rpm for 12 minutes at 25ºC.
- Discard the supernatant, pool the cells, and add **20 mL** of sterile ammonium chloride to the pooled cells.
- Place the cells on ice for 10 minutes and centrifuge at 1200 rpm for 10 minutes at 25ºC.

- Discard the supernatant, vortex the pellet, and re-suspend the cells in **1 - 2mL** of RPMI-1640.
- Count the cells microscopically, using a haemocytometer (1:10 ratio of cells: gentian violet solution).
- Based on the cell count, re-suspend the cells in a volume of RPMI-1640 that gives a final concentration of **10^7 cells/mL.**

Protocol for determining granulocyte and MNL cell viability

- Aliquot **50 µL** of each sample containing the isolated granulocytes or MNL cells into flow tubes.
- Add **1 mL** of Propidium Iodide into each flow tube and run each sample on the CytoFLEX S cytometer using the appropriate protocol for each cell type.

  NB: Cell viability is calculated in percentages.

- *Identification of different cell types in MNL cell population by flow cytometry*
- Aliquot **50 µL** of sample containing the isolated MNL cells into a flow tube.
- Add **5 µL** of each antibody (CD4-AF 700, CD3-FITC, CD8-PE, CD14-ECD) and **30 µL** of PBS to the tube.
- Incubate the tube in the dark for 15 minutes and add **1 mL** of PBS to the tube.
- Centrifuge the tube at 500 xg for 5 minutes at 25ºC.
- Discard the supernatant and re-suspend the pellet in **1 mL** of PBS.
- Run the sample on the CytoFLEX S cytometer, using the appropriate protocol.

Protocol for opsonophagocytic assay using human granulocytes and MNL cells exposed to *M. tuberculosis*, for cytokine expression

- Mix the granulocytes or MNLs together with mAb JG7 at 37ºC for 20 minutes.
- Incubate the mixture in a 5% CO_2 incubator, prior to the addition of either *M. tuberculosis* strain H37Rv or Lipopolysaccharide (LPS).
- After bacteria and LPS addition, incubate the cells for an additional 24 hours at 37ºC in a 5% CO_2 incubator.
- After 24 hours, transfer the well contents to 5 mL sterile tubes and centrifuge at 480 xg for 20 minutes at 25ºC.

- Draw the supernatants into clean eppendorf tubes and freeze at -80ºC until ready for use in the multiplex suspension array assay.

Protocol for determining cytokine expression

Sample preparation for multiplex assay

- Filter-sterilize the supernatants using 0.22 µm sterile filters before use.

Multiplex assay procedure

- Use all reagents at room temperature.
- Dispense **200 µL** of wash buffer into each well of a 96-well plate.
- Seal the plate with sealing foil and place on a shaker for 10 minutes at room temperature.
- Decant the wash buffer and dispense **25 µL** of each standard or control into appropriate wells according to a pre-designed plate map.
- Add **25 µL** of assay buffer into sample wells and **25 µL** of matrix solution (RPMI-1640) to the background, standard and control wells.
- Add **25 µL** of sample (supernatant) into the appropriate wells according to the plate map.
- Vortex each cytokine bead for 1 minute and combine the cytokine beads into one bottle.
- Vortex mixing bottle and add **25 µL** of pre-mixed cytokine beads to each well of the plate.
- Seal the plate and incubate with agitation (300 rpm) for 2 hours at room temperature.
- Wash the plate twice using an automated magnetic plate washer.
- Add **25 µL** of detection antibody per well, seal and incubate the plate with agitation (300 rpm) for 1 hour at room temperature.
- Add **25 µL** of Streptavidin-PE to each well, seal and incubate the plate with agitation (300 rpm) for 30 minutes at room temperature.
- Wash the plate twice and dispense **150 µL** of sheath fluid into each well.
- Shake the plate for 5 minutes and read on the Luminex ^{200TM} system (100 µL, 50 beads per bead set).

List of Suppliers

- Becton Dickinson, New Jersey, United States of America.
- Thermo Fisher Scientific, Massachusetts, United States of America.
- Lasec, Cape Town, South Africa.
- Biocom Diagnostics, Centurion, South Africa.
- Sigma-Aldrich GmbH, Munich, Germany.
- Longhorn Vaccines and Diagnostics, Bethesda, United States of America.
- Merck KGaA, Darmstadt, Germany.
- Bio-Rad, California, United States of America.
- ESCO Technologies, Centurion, South Africa.
- Reichert Technologies, New York, United States of America.
- Beckman Coulter, California, United States of America.
- Labotec, Midrand, South Africa.
- Zeiss, Cape Town, South Africa.

Appendix C: Data sheets for opsonophagocytic assays

Sheet 1: Raw data for *M. tuberculosis* H37Ra in control and sample wells for OP assays at various JG7 concentrations with U-937 macrophage cells

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
1	H37Ra	100	0	0	180	47	145	188	512	86
1	H37Ra	100	1	0.25	236	39	158			
1	H37Ra	100	2	0.5	166	91	98			
1	H37Ra	100	3	1	220	287	179			
1	H37Ra	100	4	2.5	189	0	296			
1	H37Ra	100	5	5	223	0	101			
1	H37Ra	100	6	10	41	73	117			
1	H37Ra	100	7	15	206	173	213			
1	H37Ra	100	8	25	21	82	50			
1	H37Ra	1000	0	0	0	22	66	249	94	0
1	H37Ra	1000	1	0.25	0	0	0			
1	H37Ra	1000	2	0.5	69	77	0			
1	H37Ra	1000	3	1	61	105	0			
1	H37Ra	1000	4	2.5	0	3	215			
1	H37Ra	1000	5	5	0	32	0			
1	H37Ra	1000	6	10	0	105	22			
1	H37Ra	1000	7	15	0	0	0			
1	H37Ra	1000	8	25	0	0	0			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
2	H37Ra	1000	0	0	770	634	567	474	245	356
2	H37Ra	1000	1	0.25	443	460	427			
2	H37Ra	1000	2	0.5	548	478	395			
2	H37Ra	1000	3	1	630	517	459			
2	H37Ra	1000	4	2.5	265	310	200			
2	H37Ra	1000	5	10	339	250	200			
2	H37Ra	1000	6	25	500	517	346			
2	H37Ra	1000	7	50	508	404	634			
2	H37Ra	1000	8	100	506	325	380			
2	H37Ra	10000	0	0	206	260	319	110	89	200
2	H37Ra	10000	1	0.25	118	60	68			
2	H37Ra	10000	2	0.5	122	155	70			
2	H37Ra	10000	3	1	85	105	42			
2	H37Ra	10000	4	2.5	100	142	92			
2	H37Ra	10000	5	10	117	152	133			
2	H37Ra	10000	6	25	125	125	121			
2	H37Ra	10000	7	50	272	239	215			
2	H37Ra	10000	8	100	130	126	197			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
3	H37Ra	100	0	0	148	89	135	148	90	92
3	H37Ra	100	1	0.25	122	109	97			
3	H37Ra	100	2	0.5	106	121	121			
3	H37Ra	100	3	1	154	138	120			
3	H37Ra	100	4	2.5	104	137	134			
3	H37Ra	100	5	10	110	112	99			
3	H37Ra	100	6	25	86	85	89			
3	H37Ra	100	7	50	168	113	126			
3	H37Ra	100	8	100	90	68	167			
3	H37Ra	1000	0	0	20	13	28	22	20	10
3	H37Ra	1000	1	0.25	10	6	6			
3	H37Ra	1000	2	0.5	22	4	3			
3	H37Ra	1000	3	1	5	21	11			
3	H37Ra	1000	4	2.5	24	15	0			
3	H37Ra	1000	5	10	23	4	20			
3	H37Ra	1000	6	25	11	0	26			
3	H37Ra	1000	7	50	12	115	29			
3	H37Ra	1000	8	100	32	31	10			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
4	H37Ra	1000	0	0	42	53	27	115	53	63
4	H37Ra	1000	1	0.25	163	60	78			
4	H37Ra	1000	2	0.5	75	157	115			
4	H37Ra	1000	3	1	14	119	178			
4	H37Ra	1000	4	2.5	24	98	54			
4	H37Ra	1000	5	10	189	70	60			
4	H37Ra	1000	6	25	107	118	86			
4	H37Ra	1000	7	50	30	50	35			
4	H37Ra	1000	8	100	69	110	57			
4	H37Ra	10000	0	0	147	64	70	84	67	87
4	H37Ra	10000	1	0.25	89	78	80			
4	H37Ra	10000	2	0.5	93	87	92			
4	H37Ra	10000	3	1	48	30	20			
4	H37Ra	10000	4	2.5	75	26	5			
4	H37Ra	10000	5	10	51	50	59			
4	H37Ra	10000	6	25	34	47	60			
4	H37Ra	10000	7	50	22	25	13			
4	H37Ra	10000	8	100	30	29	57			

Sheet 2: Raw data for clinical, susceptible TB (CMTB) in control and sample wells for OP assays at various JG7 concentrations with U-937 macrophage cells

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
1	CMTB	100	0	0	267	264	441	485	363	383
1	CMTB	100	1	0.25	331	512	529			
1	CMTB	100	2	0.5	317	226	315			
1	CMTB	100	3	1	354	261	201			
1	CMTB	100	4	2.5	334	343	244			
1	CMTB	100	5	5	269	269	308			
1	CMTB	100	6	10	246	264	296			
1	CMTB	100	7	15	162	264	256			
1	CMTB	100	8	25	117	164	267			
1	CMTB	1000	0	0	264	479	407	426	368	
1	CMTB	1000	1	0.25	339	238	176			
1	CMTB	1000	2	0.5	321	238	227			
1	CMTB	1000	3	1	305	245	186			
1	CMTB	1000	4	2.5	391	214	126			
1	CMTB	1000	5	5	297	493	230			
1	CMTB	1000	6	10	425	369	381			
1	CMTB	1000	7	15	378	393	282			
1	CMTB	1000	8	25	332	340	322			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
2	CMTB	100	0	0	157	213	240	520	550	211
2	CMTB	100	1	0.25	182	155	176			
2	CMTB	100	2	0.5	142	140	142			
2	CMTB	100	3	1	199	322	137			
2	CMTB	100	4	2.5	200	131	130			
2	CMTB	100	5	10	144	149	141			
2	CMTB	100	6	25	140	140	150			
2	CMTB	100	7	50	166	176	140			
2	CMTB	100	8	100	184	152	138			
2	CMTB	1000	0	0	205	345	354	252	211	162
2	CMTB	1000	1	0.25	139	140	134			
2	CMTB	1000	2	0.5	111	110	102			
2	CMTB	1000	3	1	82	144	74			
2	CMTB	1000	4	2.5	140	138	126			
2	CMTB	1000	5	10	116	129	150			
2	CMTB	1000	6	25	102	95	130			
2	CMTB	1000	7	50	119	86	102			
2	CMTB	1000	8	100	133	180	160			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
3	CMTB	100	0	0	916	905	813	938	555	1068
3	CMTB	100	1	0.25	1047	1030	960			
3	CMTB	100	2	0.5	807	781	919			
3	CMTB	100	3	1	760	972	890			
3	CMTB	100	4	2.5	662	954	815			
3	CMTB	100	5	10	646	754	614			
3	CMTB	100	6	25	869	1000	684			
3	CMTB	100	7	50	770	751	859			
3	CMTB	100	8	100	990	1058	867			
3	CMTB	1000	0	0	375	371	356	474	575	528
3	CMTB	1000	1	0.25	470	336	405			
3	CMTB	1000	2	0.5	466	364	357			
3	CMTB	1000	3	1	329	459	498			
3	CMTB	1000	4	2.5	303	588	417			
3	CMTB	1000	5	10	357	522	319			
3	CMTB	1000	6	25	271	490	404			
3	CMTB	1000	7	50	283	376	366			
3	CMTB	1000	8	100	433	474	385			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
4	CMTB	1000	0	0	694	366	820	700	890	662
4	CMTB	1000	1	0.25	692	807	768			
4	CMTB	1000	2	0.5	730	693	1002			
4	CMTB	1000	3	1	743	780	599			
4	CMTB	1000	4	2.5	577	600	742			
4	CMTB	1000	5	10	719	564	561			
4	CMTB	1000	6	25	495	371	591			
4	CMTB	1000	7	50	611	638	500			
4	CMTB	1000	8	100	950	873	760			
4	CMTB	10000	0	0	405	734	609	618	538	570
4	CMTB	10000	1	0.25	500	608	550			
4	CMTB	10000	2	0.5	376	540	383			
4	CMTB	10000	3	1	432	377	524			
4	CMTB	10000	4	2.5	519	409	590			
4	CMTB	10000	5	10	376	525	439			
4	CMTB	10000	6	25	479	457	478			
4	CMTB	10000	7	50	431	598	321			
4	CMTB	10000	8	100	345	412	493			

Sheet 3: Raw data for *M. tuberculosis* H37Ra in control and sample wells for OP assays at various JG7 concentrations with HL-60 granulocyte cells

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
1	H37Ra	100	0	0	702	649	496	465	613	362
1	H37Ra	100	1	0.25	401	474	337			
1	H37Ra	100	2	0.5	417	463	315			
1	H37Ra	100	3	1	222	263	275			
1	H37Ra	100	4	2.5	403	300	570			
1	H37Ra	100	5	10	628	455	611			
1	H37Ra	100	6	25	746	380	467			
1	H37Ra	100	7	50	204	433	307			
1	H37Ra	100	8	100	186	521	109			
1	H37Ra	1000	0	0	367	260	376	182	217	217
1	H37Ra	1000	1	0.25	344	187	249			
1	H37Ra	1000	2	0.5	128	176	173			
1	H37Ra	1000	3	1	162	221	167			
1	H37Ra	1000	4	2.5	300	319	313			
1	H37Ra	1000	5	10	246	262	227			
1	H37Ra	1000	6	25	186	182	256			
1	H37Ra	1000	7	50	159	200	246			
1	H37Ra	1000	8	100	175	176	208			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
2	H37Ra	100	0	0	391	411	167	391	415	634
2	H37Ra	100	1	0.25	427	650	466			
2	H37Ra	100	2	0.5	467	366	322			
2	H37Ra	100	3	1	58	368	377			
2	H37Ra	100	4	2.5	534	99	378			
2	H37Ra	100	5	10	390	428	400			
2	H37Ra	100	6	25	397	347	442			
2	H37Ra	100	7	50	252	367	128			
2	H37Ra	100	8	100	478	488	173			
2	H37Ra	1000	0	0	212	337	243	217	211	223
2	H37Ra	1000	1	0.25	299	271	370			
2	H37Ra	1000	2	0.5	210	187	195			
2	H37Ra	1000	3	1	214	244	222			
2	H37Ra	1000	4	2.5	189	237	419			
2	H37Ra	1000	5	10	236	233	209			
2	H37Ra	1000	6	25	277	372	343			
2	H37Ra	1000	7	50	126	149	119			
2	H37Ra	1000	8	100	251	223	214			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
3	H37Ra	1000	0	0	654	745	660	650	511	530
3	H37Ra	1000	1	0.25	423	725	758			
3	H37Ra	1000	2	0.5	744	947	705			
3	H37Ra	1000	3	1	582	520	752			
3	H37Ra	1000	4	2.5	635	664	437			
3	H37Ra	1000	5	10	735	421	407			
3	H37Ra	1000	6	25	662	737	759			
3	H37Ra	1000	7	50	519	467	520			
3	H37Ra	1000	8	100	438	324	484			
3	H37Ra	10000	0	0	440	368	350	520	300	443
3	H37Ra	10000	1	0.25	230	165	331			
3	H37Ra	10000	2	0.5	430	314	250			
3	H37Ra	10000	3	1	351	300	369			
3	H37Ra	10000	4	2.5	304	274	282			
3	H37Ra	10000	5	10	373	500	450			
3	H37Ra	10000	6	25	415	322	419			
3	H37Ra	10000	7	50	328	315	493			
3	H37Ra	10000	8	100	343	325	477			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
4	H37Ra	1000	0	0	517	362	483	344	378	572
4	H37Ra	1000	1	0.25	321	420	623			
4	H37Ra	1000	2	0.5	286	277	259			
4	H37Ra	1000	3	1	390	197	391			
4	H37Ra	1000	4	2.5	547	354	588			
4	H37Ra	1000	5	10	838	634	531			
4	H37Ra	1000	6	25	605	772	805			
4	H37Ra	1000	7	50	347	378	534			
4	H37Ra	1000	8	100	371	319	440			
4	H37Ra	10000	0	0	435	613	612	385	413	520
4	H37Ra	10000	1	0.25	426	271	536			
4	H37Ra	10000	2	0.5	219	223	247			
4	H37Ra	10000	3	1	166	155	422			
4	H37Ra	10000	4	2.5	353	648	566			
4	H37Ra	10000	5	10	219	265	198			
4	H37Ra	10000	6	25	707	978	682			
4	H37Ra	10000	7	50	106	190	137			
4	H37Ra	10000	8	100	522	183	319			

Sheet 4: Raw data for clinical, susceptible TB (CMTB) in control and sample wells for OP assays at various JG7 concentrations with HL-60 granulocyte cells

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
1	CMTB	100	0	0	719	562	526	498	420	373
1	CMTB	100	1	0.25	397	426	278			
1	CMTB	100	2	0.5	515	461	535			
1	CMTB	100	3	1	415	421	454			
1	CMTB	100	4	2.5	348	398	412			
1	CMTB	100	5	10	441	573	361			
1	CMTB	100	6	25	652	601	603			
1	CMTB	100	7	50	500	528	506			
1	CMTB	100	8	100	598	515	570			
1	CMTB	1000	0	0	291	230	210	306	264	244
1	CMTB	1000	1	0.25	208	239	213			
1	CMTB	1000	2	0.5	253	262	150			
1	CMTB	1000	3	1	236	227	221			
1	CMTB	1000	4	2.5	280	179	292			
1	CMTB	1000	5	10	169	348	278			
1	CMTB	1000	6	25	318	307	269			
1	CMTB	1000	7	50	336	300	239			
1	CMTB	1000	8	100	260	251	290			

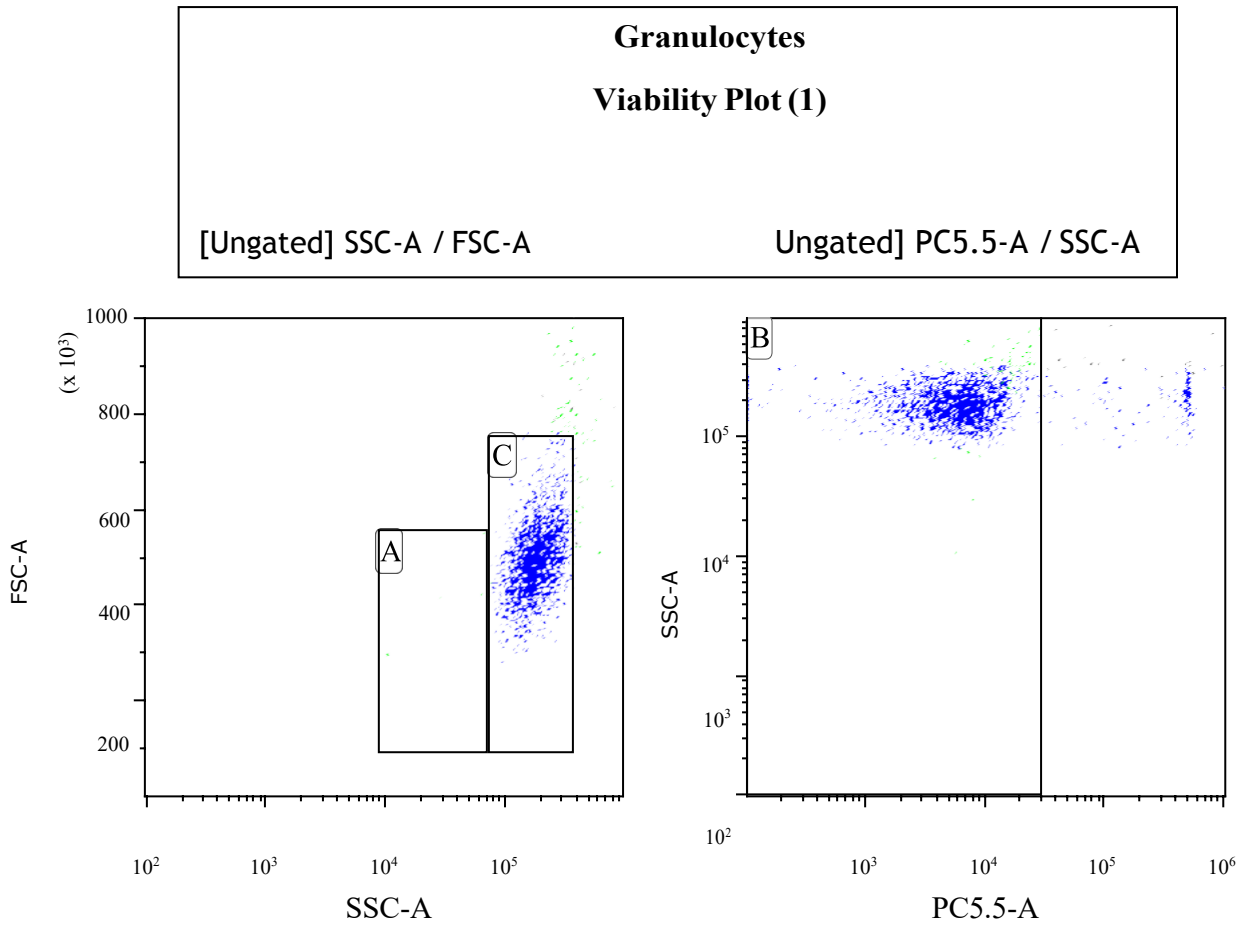
Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
2	CMTB	100	0	0	1195	1160	1082	720	967	934
2	CMTB	100	1	0.25	913	968	428			
2	CMTB	100	2	0.5	935	847	903			
2	CMTB	100	3	1	621	920	489			
2	CMTB	100	4	2.5	1060	1175	1103			
2	CMTB	100	5	10	595	678	865			
2	CMTB	100	6	25	842	940	741			
2	CMTB	100	7	50	945	925	836			
2	CMTB	100	8	100	1015	1164	703			
2	CMTB	1000	0	0	983	586	612	548	456	591
2	CMTB	1000	1	0.25	612	970	880			
2	CMTB	1000	2	0.5	738	717	638			
2	CMTB	1000	3	1	649	630	786			
2	CMTB	1000	4	2.5	775	845	603			
2	CMTB	1000	5	10	414	531	228			
2	CMTB	1000	6	25	920	986	752			
2	CMTB	1000	7	50	672	553	710			
2	CMTB	1000	8	100	714	697	700			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
3	CMTB	1000	0	0	572	581	586	377	495	417
3	CMTB	1000	1	0.25	656	783	653			
3	CMTB	1000	2	0.5	626	722	667			
3	CMTB	1000	3	1	495	500	533			
3	CMTB	1000	4	2.5	365	365	308			
3	CMTB	1000	5	10	561	550	629			
3	CMTB	1000	6	25	727	636	669			
3	CMTB	1000	7	50	425	430	552			
3	CMTB	1000	8	100	344	327	490			
3	CMTB	10000	0	0	538	348	340	453	469	294
3	CMTB	10000	1	0.25	518	400	350			
3	CMTB	10000	2	0.5	599	617	281			
3	CMTB	10000	3	1	301	337	408			
3	CMTB	10000	4	2.5	278	270	277			
3	CMTB	10000	5	10	403	354	466			
3	CMTB	10000	6	25	506	464	512			
3	CMTB	10000	7	50	521	417	396			
3	CMTB	10000	8	100	310	320	180			

Experiment	TB strain	TB dilution	Treatment	JG7 conc.	repl_1	repl_2	repl_3	repl_4	repl_5	repl_6
4	CMTB	100	0	0	719	534	461	331	305	439
4	CMTB	100	1	0.25	536	445	320			
4	CMTB	100	2	0.5	350	380	383			
4	CMTB	100	3	1	474	438	410			
4	CMTB	100	4	2.5	459	457	276			
4	CMTB	100	5	10	602	507	547			
4	CMTB	100	6	25	542	496	410			
4	CMTB	100	7	50	618	596	531			
4	CMTB	100	8	100	634	483	478			
4	CMTB	1000	0	0	193	203	171	156	188	148
4	CMTB	1000	1	0.25	178	164	159			
4	CMTB	1000	2	0.5	187	212	143			
4	CMTB	1000	3	1	165	170	197			
4	CMTB	1000	4	2.5	129	147	181			
4	CMTB	1000	5	10	187	179	216			
4	CMTB	1000	6	25	198	223	181			
4	CMTB	1000	7	50	157	209	162			
4	CMTB	1000	8	100	176	171	220			

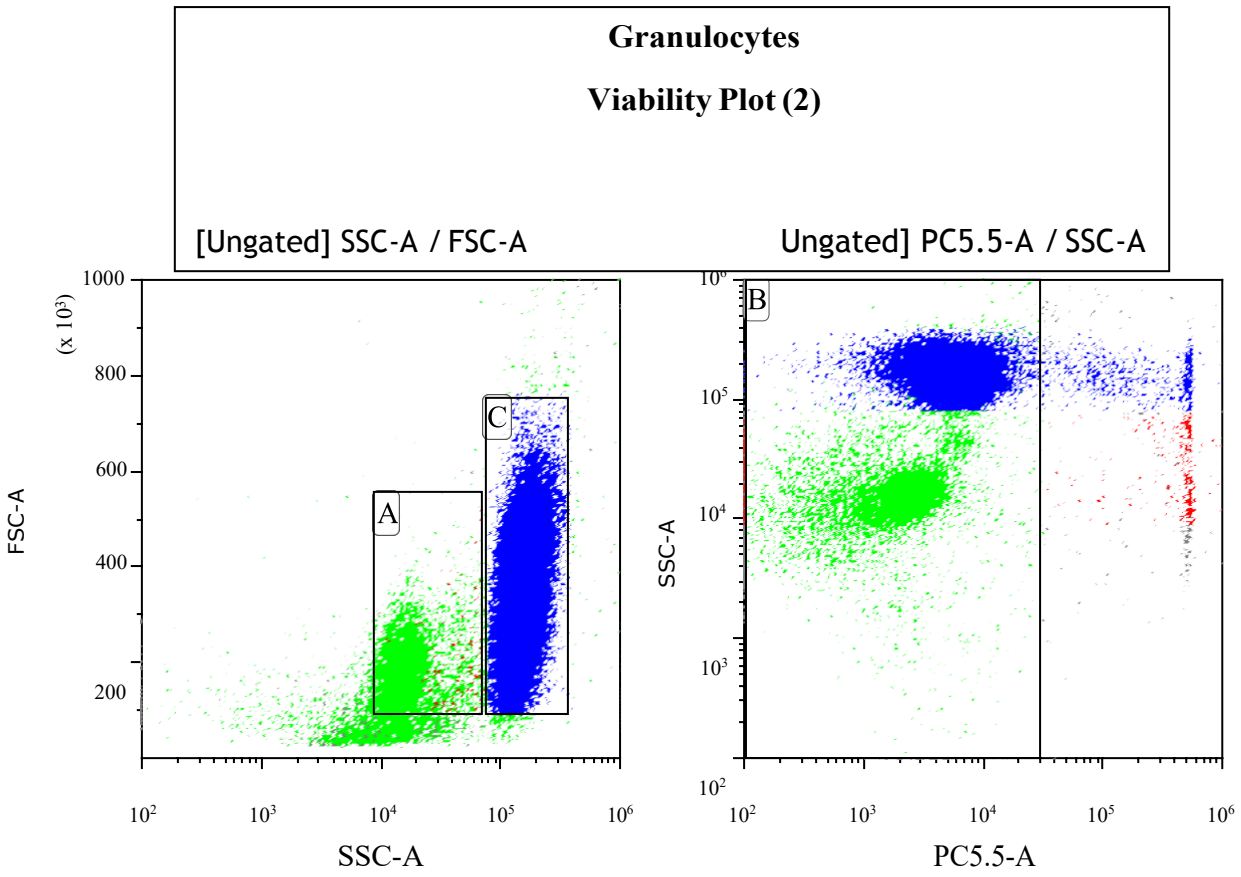
Appendix D: Data sheets for multiplex immunoassay

Sheet 1: Granulocyte viability plots



Gate	Number	%Total	%Gated	Cells/µL
All	2 342	100.00	100.00	23
A	5	0.21	0.21	0
C	2 243	95.77	95.77	23

Gate	Number	%Total	%Gated
All	2 342	100.00	100.00
B	2 095	89.45	89.45



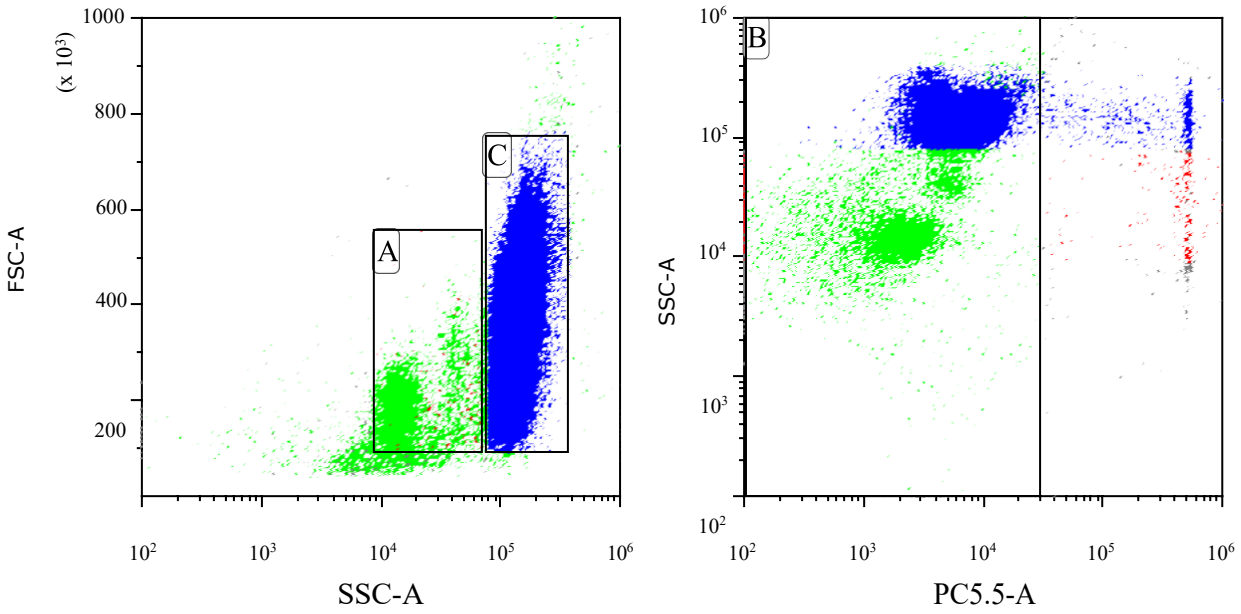
Gate	Number	%Total	%Gated	Cells/µL	Gate	Number	%Total	%Gated
All	62 419	100.00	100.00	624	All	62 419	100.00	100.00
A	7 854	12.58	12.58	79	B	59 932	96.02	96.02
C	50 058	80.20	80.20	501				

Granulocytes

Viability Plot (3)

[Ungated] SSC-A / FSC-A

Ungated] PC5.5-A / SSC-A

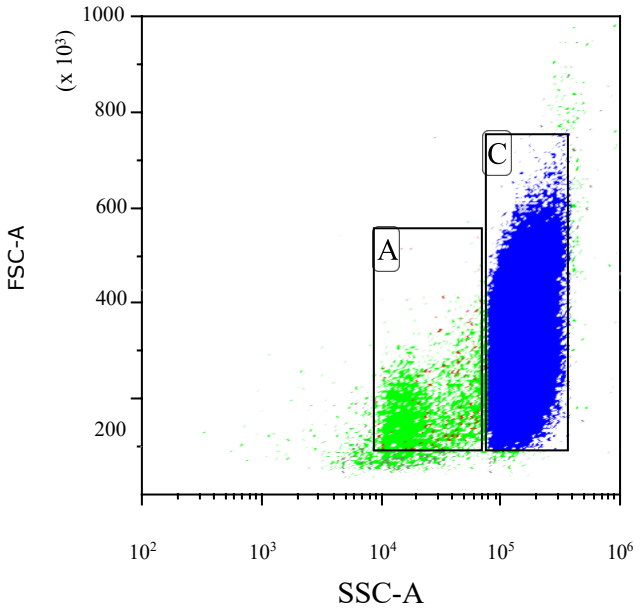


Gate	Number	%Total	%Gated	Cells/µL	Gate	Number	%Total	%Gated
All	66 391	100.00	100.00	664	All	66 391	100.00	100.00
A	6 189	9.32	9.32	62	B	64 976	97.87	97.87
C	57 857	87.15	87.15	579				

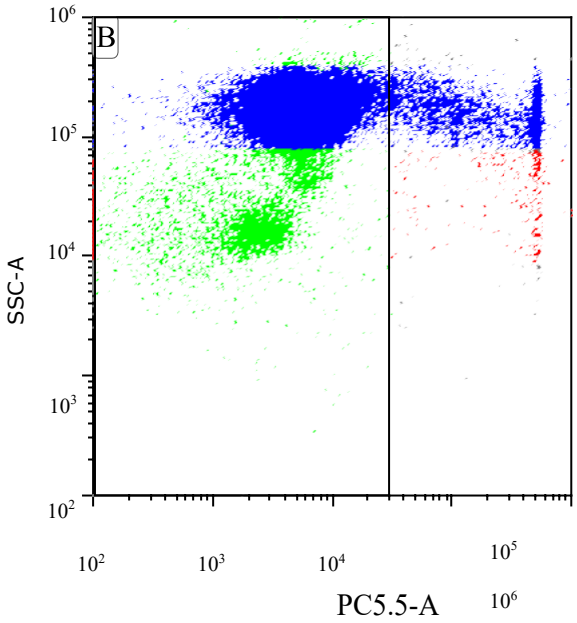
Granulocytes

Viability Plot (4)

[Ungated] SSC-A / FSC-A



Ungated] PC5.5-A / SSC-A



Gate	Number	%Total	%Gated	Cells/µL
All	95 757	100.00	100.00	958
A	4 410	4.61	4.61	44
C	89 683	93.66	93.66	897

Gate	Number	%Total	%Gated
All	95 757	100.00	100.00
B	91 330	95.38	95.38

Sheet 2: Raw data obtained for granulocytes with multiplex suspension array assay

Type	Description	Group	Cell type	TB strain	JG7 conc.	LPS	IL-1β	IL-6	IL-8	IL-10	IL-12p70	IFN-γ	TNF-α
X1	05/12 Neutro Cont 1	1	Granulocyte			0	226.32	4144.45	11633.86	312.09	49.34	OOR <	523.97
X2	Neutro EC 1	1	Granulocyte	H37Rv		0	1269.53	4121.45	12193.12	344.78	38.76	OOR <	1409.17
X3	Neutro E 1	1	Granulocyte	H37Rv	25 µg/mL	0	1406.46	5916.36	24753.52	407.32	38.76	OOR <	1485.8
X4	Neutro LPS 1	1	Granulocyte			1	1274.14	12686.56	14509.14	898.87	104.72	OOR <	1524.48
X5	05/12 Neutro Cont 2	1	Granulocyte			0	237.49	3768.63	13211.2	333.64	38.76	OOR <	566.11
X6	Neutro EC 2	1	Granulocyte	H37Rv		0	1204.58	4714.8	13283.23	384.63	54.19	OOR <	1459.22
X7	Neutro E 2	1	Granulocyte	H37Rv	25 µg/mL	0	1451.37	6093.12	OOR >	425.61	38.76	OOR <	1609.49
X8	Neutro LPS 2	1	Granulocyte			1	1188.36	10883.97	22907.37	870.7	90.84	OOR <	1541.95
X9	09/12 Neutro Cont 1	2	Granulocyte			0	125.24	887.11	11695.8	173.38	38.76	OOR <	186.06
X10	Neutro EC 1	2	Granulocyte	H37Rv		0	395.26	2568.14	11459.74	277.02	26.35	92.18	1157.97
X11	Neutro E 1	2	Granulocyte	H37Rv	25 µg/mL	0	542.28	4287.12	11758.46	573.5	26.35	OOR <	1221.09
X12	Neutro LPS 1	2	Granulocyte			1	624.85	6590.57	19664.9	991.82	49.34	OOR <	518.6
X13	09/12 Neutro Cont 2	2	Granulocyte			0	138.66	834.07	8302.09	150.91	9.06	OOR <	190.31
X14	Neutro EC 2	2	Granulocyte	H37Rv		0	378.56	2811.08	9513.18	284.88	26.35	92.18	1143.44
X15	Neutro E 2	2	Granulocyte	H37Rv	25 µg/mL	0	586.29	4833.49	14794.78	574.5	9.06	OOR <	1393.85
X16	Neutro LPS 2	2	Granulocyte			1	623.43	6657.61	21854.77	968.18	49.34	OOR <	537.29
X17	10/12 Neutro Cont 1	3	Granulocyte			0	395.08	7532.83	*41096.36	837.25	9.06	OOR <	523.97
X18	Neutro EC 1	3	Granulocyte	H37Rv		0	1090.14	10205.27	*46855.96	712.56	26.35	OOR <	2224.86
X19	Neutro E 1	3	Granulocyte	H37Rv	25 µg/mL	0	1361.65	13025.96	15814.57	1003.86	9.06	252.25	2511.68
X20	Neutro LPS 1	3	Granulocyte			1	758.39	16791.47	21651.79	1963.61	26.35	OOR <	1351.19
X21	10/12 Neutro Cont 2	3	Granulocyte			0	369.9	7229.52	17698.3	833.31	9.06	OOR <	694.79
X22	Neutro EC 2	3	Granulocyte	H37Rv		0	918.02	9050.85	13729.49	749.36	9.06	92.18	2139.94
X23	Neutro E 2	3	Granulocyte	H37Rv	25 µg/mL	0	1511.03	14224.13	18699.44	1284.09	9.06	252.25	2755.85
X24	Neutro LPS 2	3	Granulocyte			1	832.59	17358.73	32651.63	1905.23	26.35	OOR <	1419.95
X25	11/12 Neutro Cont 1	4	Granulocyte			0	1132.35	5663.35	13430.03	207.77	58.82	292.41	1887.62
X26	Neutro EC 1	4	Granulocyte	H37Rv		0	1035.26	5310.24	21211.8	190.07	49.34	OOR <	2078.05
X27	Neutro E 1	4	Granulocyte	H37Rv	25 µg/mL	0	977.45	6142.06	16035.78	224.54	26.35	OOR <	2060.51
X28	Neutro LPS 1	4	Granulocyte			1	1556.8	10072.98	16306.16	543.13	123.91	OOR <	2493.41
X29	11/12 Neutro Cont 2	4	Granulocyte			0	907.45	4868.4	15676.14	209.68	49.34	207.85	1747.76
X30	Neutro EC 2	4	Granulocyte	H37Rv		0	1098.06	5470.06	14637.37	190.07	49.34	OOR <	2071.68
X31	Neutro E 2	4	Granulocyte	H37Rv	25 µg/mL	0	1005.04	6008.51	*38767.85	242.27	49.34	OOR <	2087.61
X32	Neutro LPS 2	4	Granulocyte			1	1863	12956.41	*39144.64	702.09	158.27	OOR <	2467.47

Description: This table presents the cytokine data obtained for human granulocytes following interaction with *M. tuberculosis* strain H37Rv, mAb JG7, and LPS, after 24 hours of incubation. Numbers represent observed concentrations (in pg/mL) for each cytokine, *Value = Value extrapolated beyond standard range, OOR = Out of Range, OOR< = Out of Range (Below range).

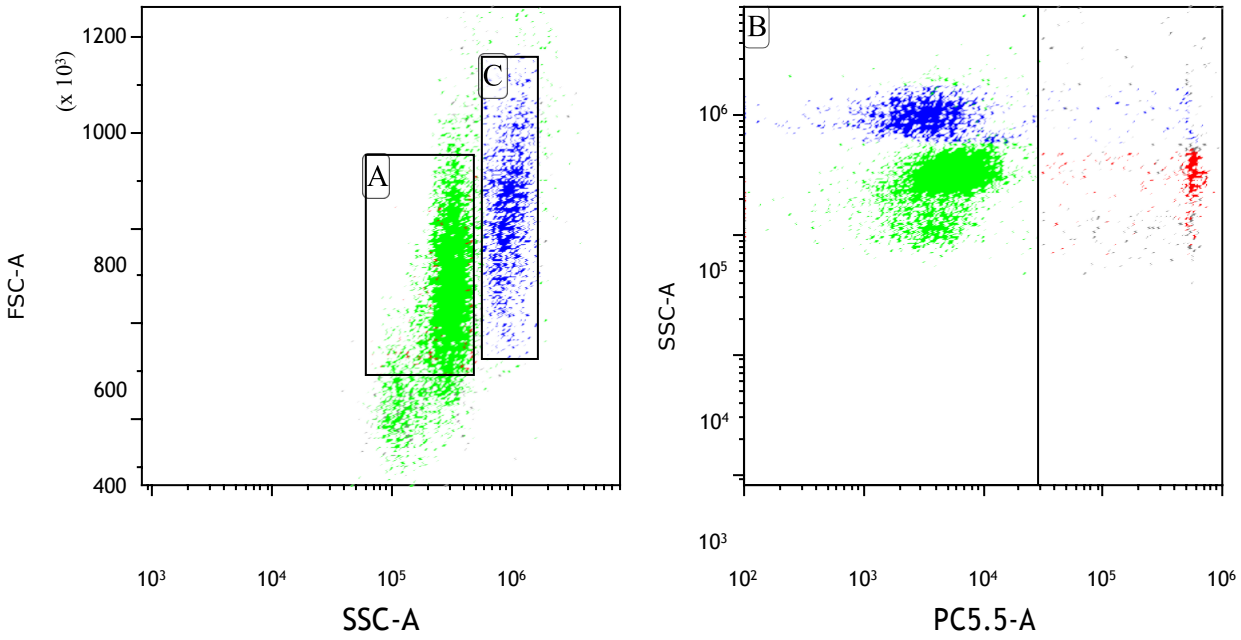
Sheet 3: MNL viability plots

MNL

Viability Plot (1)

[Ungated] SSC-A / FSC-A

Ungated] PC5.5-A / SSC-A



Gate	Number	%Total	%Gated	Cells/µL
All	10 678	100.00	100.00	107
A	6 808	63.76	63.76	68
C	2 035	19.06	19.06	20

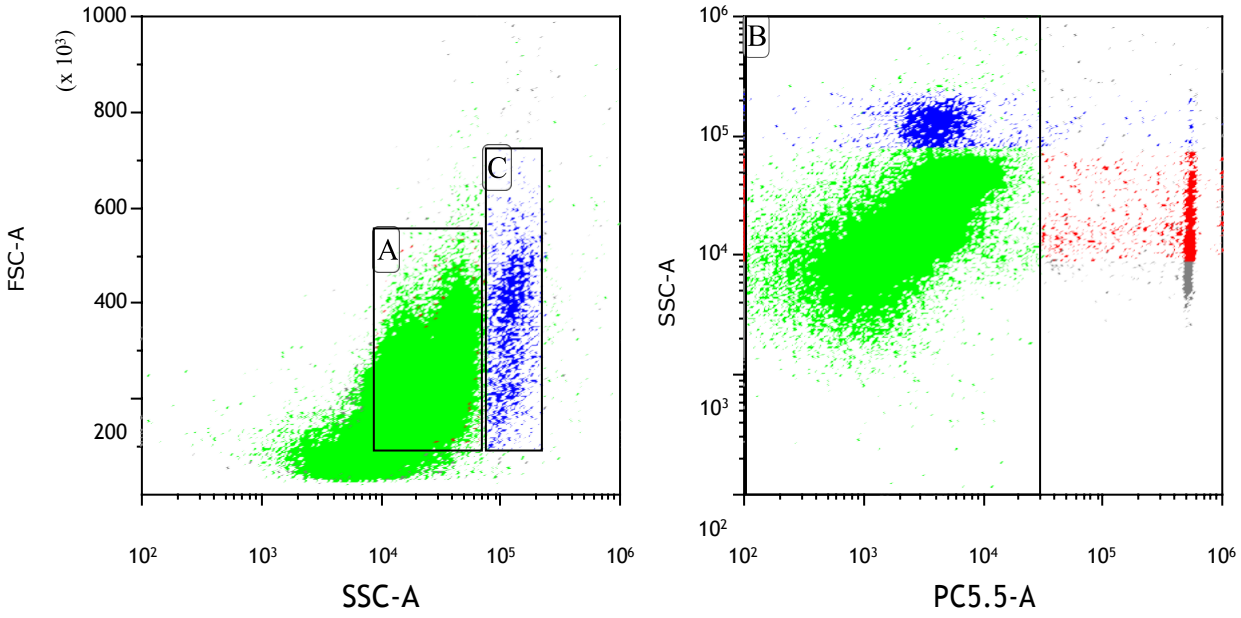
Gate	Number	%Total	%Gated
All	10 678	100.00	100.00
B	9 891	92.63	92.63

MNL

Viability Plot (2)

[Ungated] SSC-A / FSC-A

Ungated] PC5.5-A / SSC-A



Gate	Number	%Total	%Gated	Cells/µL
All	98 976	100.00	100.00	990
A	80 817	81.65	81.65	808
C	2 266	2.29	2.29	23

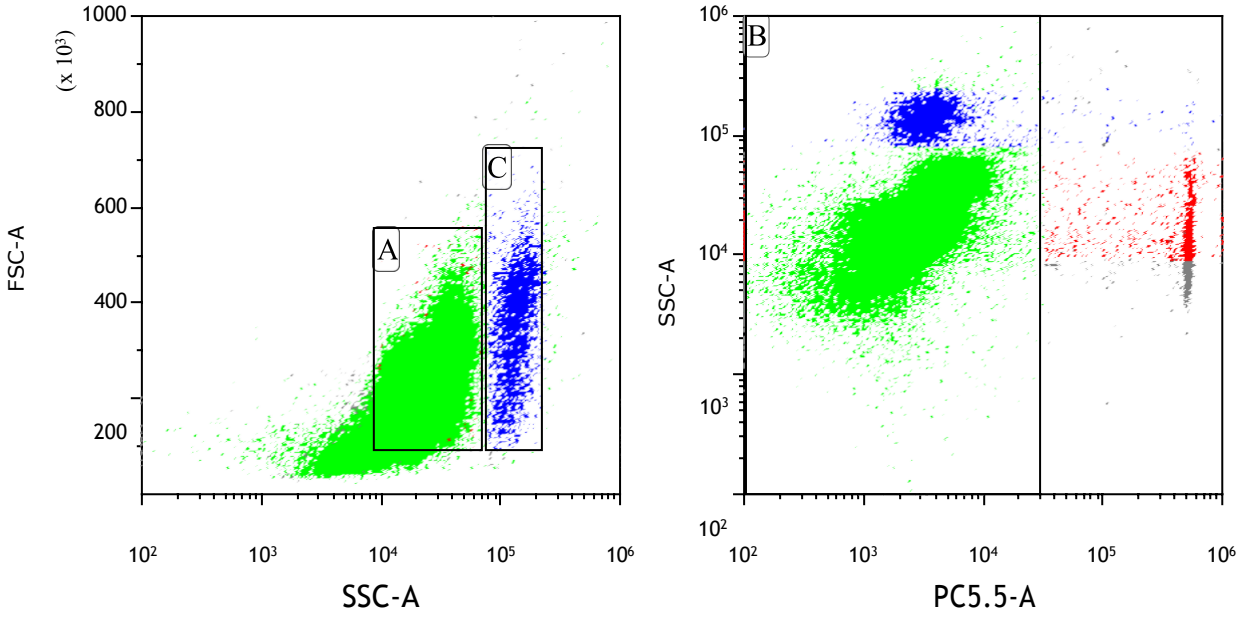
Gate	Number	%Total	%Gated
All	98 976	100.00	100.00
B	94 477	95.45	95.45

MNL

Viability Plot (3)

[Ungated] SSC-A / FSC-A

Ungated] PC5.5-A / SSC-A



Gate	Number	%Total	%Gated	Cells/µL
All	123 681	100.00	100.00	1 237
A	107 604	87.00	87.00	1 076
C	3 956	3.20	3.20	40

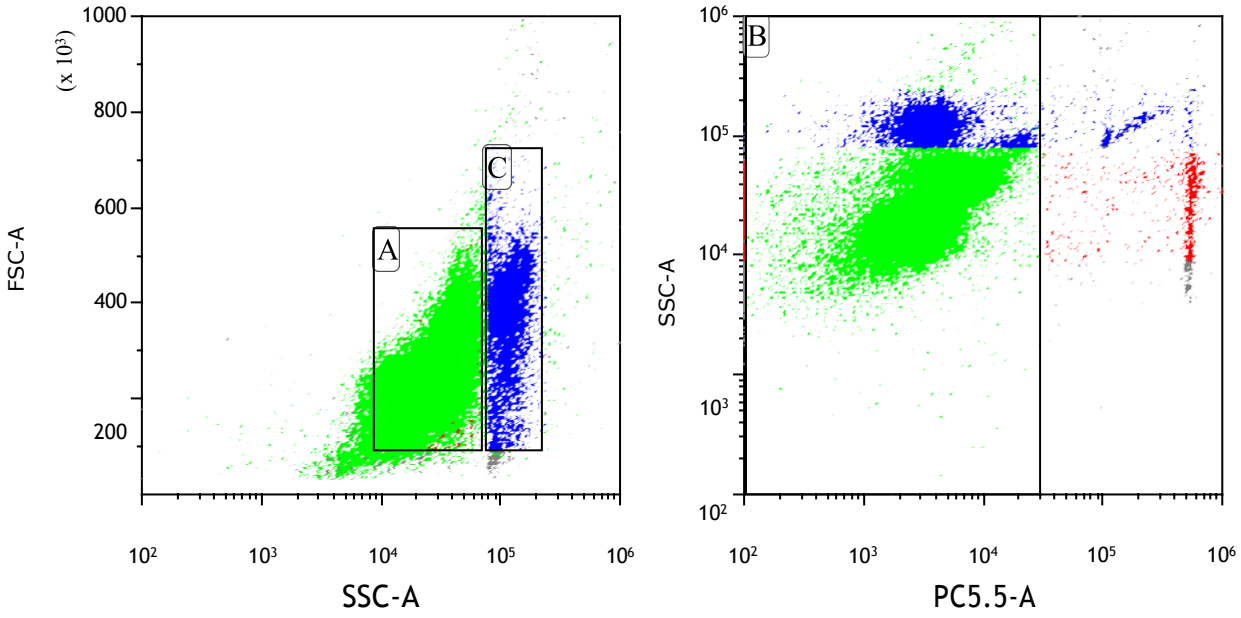
Gate	Number	%Total	%Gated
All	123 681	100.00	100.00
B	121 373	98.13	98.13

MNL

Viability Plot (4)

[Ungated] SSC-A / FSC-A

Ungated] PC5.5-A / SSC-A

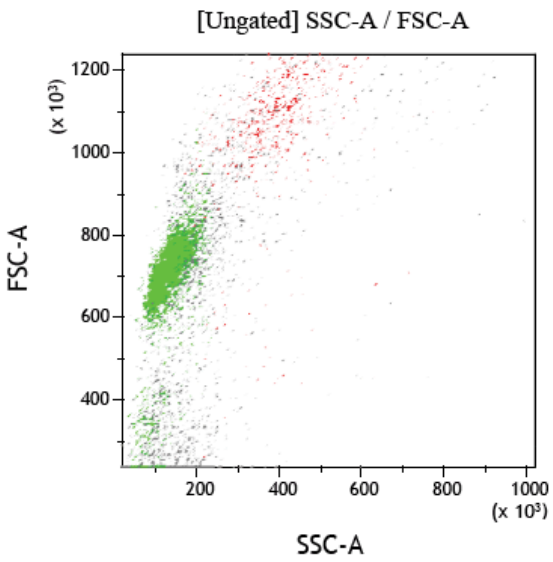


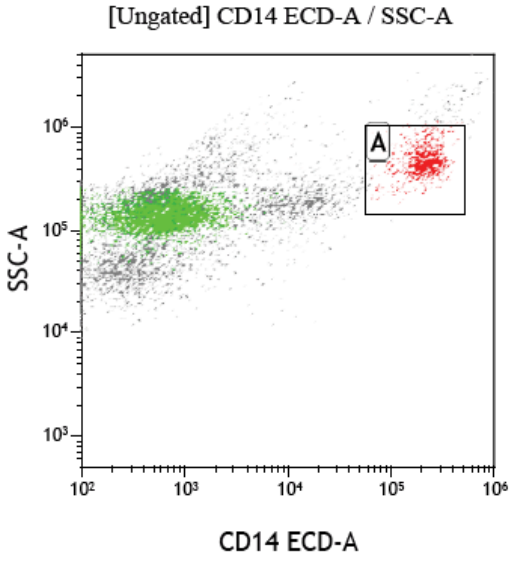
Gate	Number	%Total	%Gated	Cells/µL
All	77 322	100.00	100.00	773
A	66 453	85.94	85.94	665
C	6 826	8.83	8.83	68

Gate	Number	%Total	%Gate
All	77 322	100.00	100.00
B	74 885	96.85	96.85

Sheet 4: MNL subpopulation viability plots

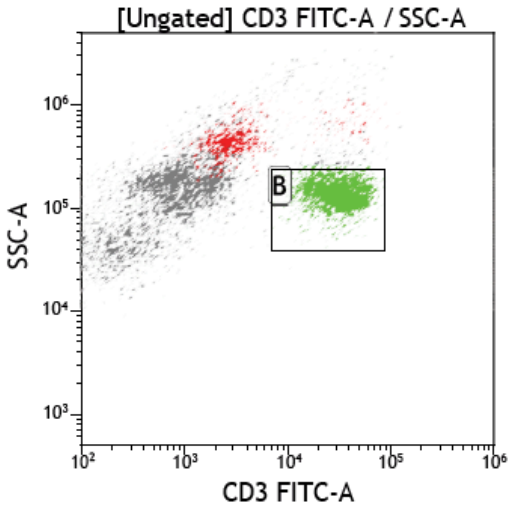
MNL sub-population 1

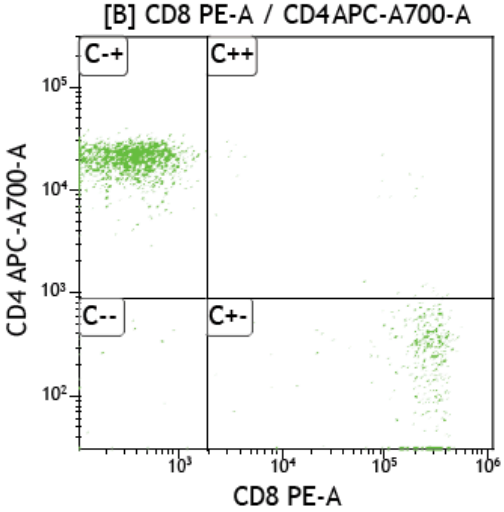




Gate	Number	%Total	%Gated
All	10 000	100.00	100.00

Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
A	785	7.85	7.85



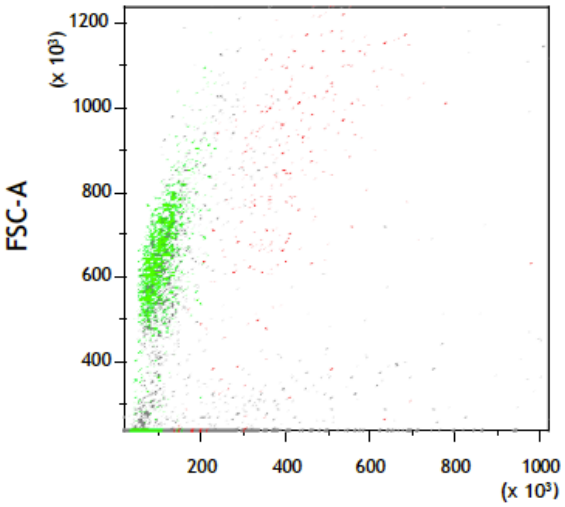


Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
B	3 391	33.91	33.91

Gate	Number	%Total	%Gated
All	3 391	33.91	100.00
C--	19	0.19	0.56
C-+	2 922	29.22	86.17
C+-	424	4.24	12.50
C++	26	0.26	0.77

MNL sub-population 2

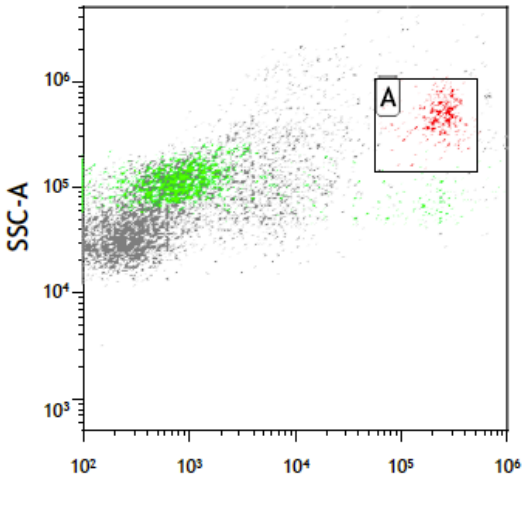
[Ungated] SSC-A / FSC-A



SSC-A

Gate	Number	%Total	%Gated
All	10 000	100.00	100.00

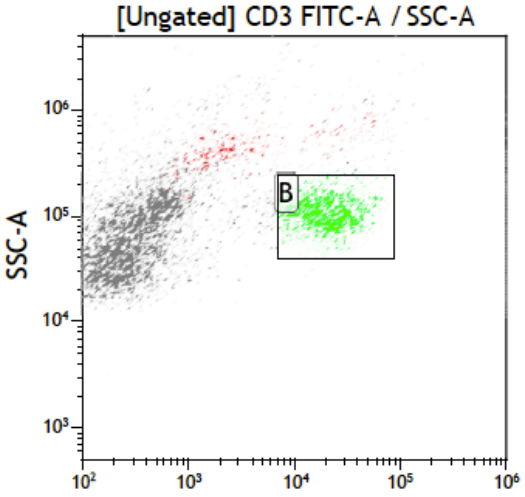
[Ungated] CD14 ECD-A / SSC-A



CD14 ECD-A

Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
A	379	3.79	3.79

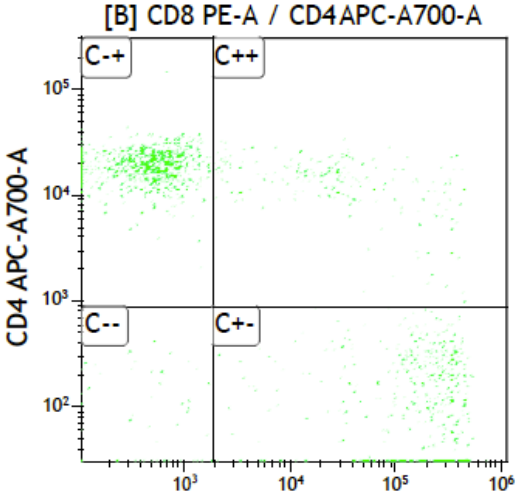
[Ungated] CD3 FITC-A / SSC-A



CD3 FITC-A

Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
B	1 638	16.38	16.38

[B] CD8 PE-A / CD4 APC-A700-A

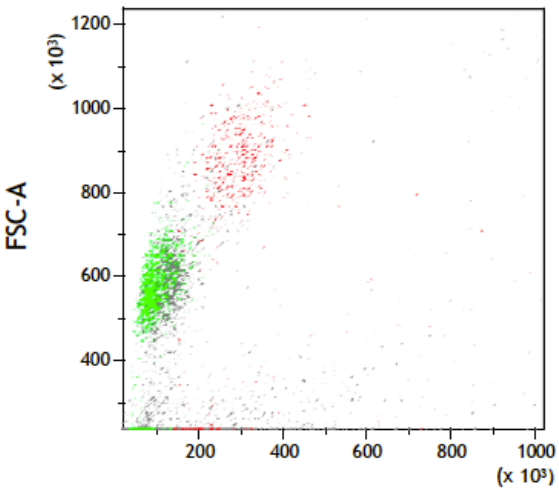


CD8 PE-A

Gate	Number	%Total	%Gated
All	1 638	16.38	100.00
C--	52	0.52	3.17
C-+	884	8.84	53.97
C+-	508	5.08	31.01
C++	194	1.94	11.84

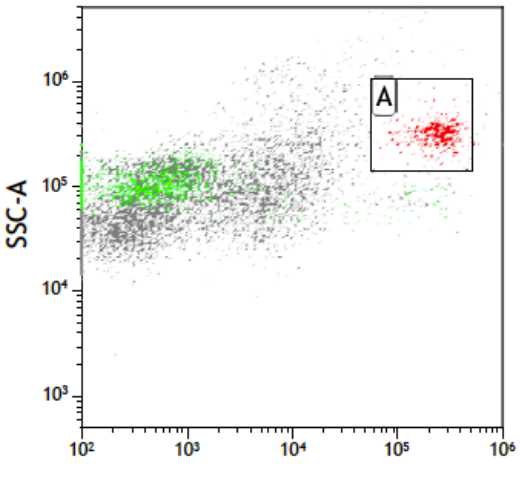
MNL sub-population 3

[Ungated] SSC-A / FSC-A



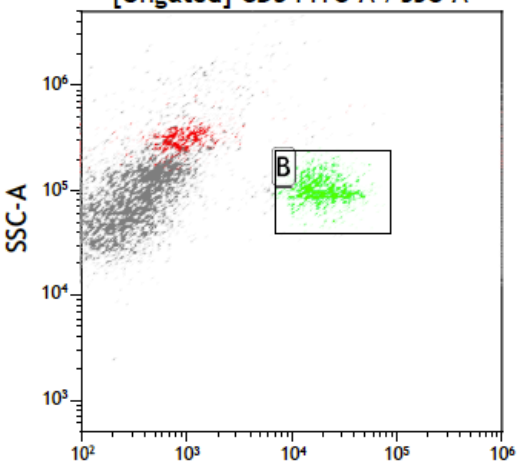
Gate	Number	%Total	%Gated
All	10 000	100.00	100.00

[Ungated] CD14 ECD-A / SSC-A



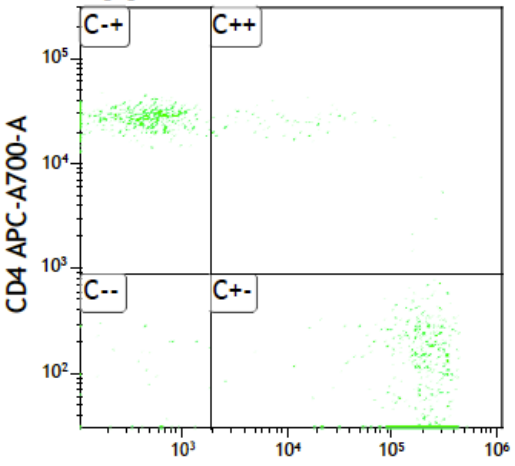
Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
A	550	5.50	5.50

[Ungated] CD3 FITC-A / SSC-A



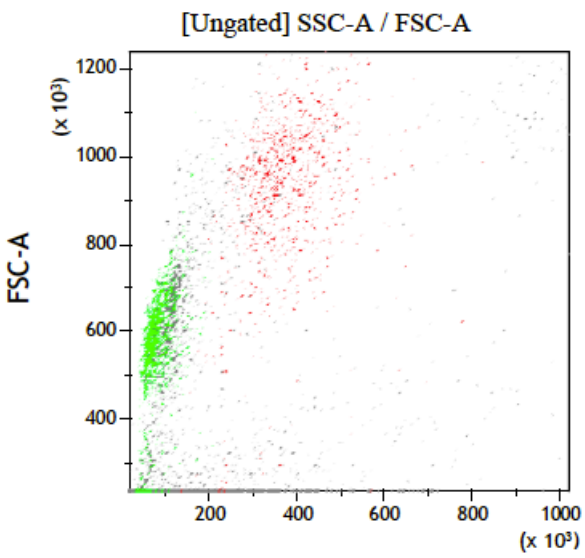
Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
B	1 221	12.21	12.21

[B] CD8 PE-A / CD4APC-A700-A

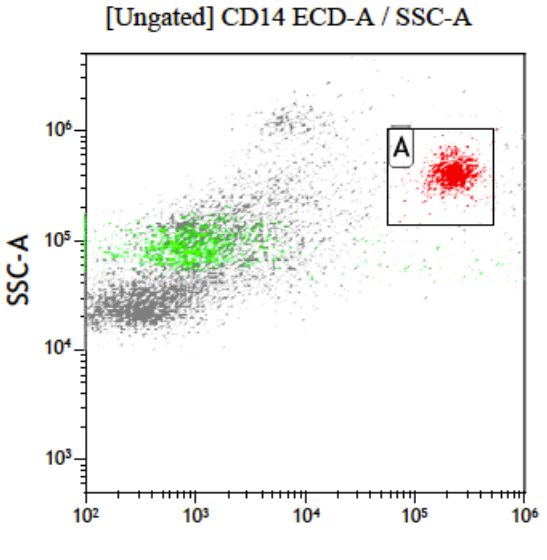


Gate	Number	%Total	%Gated
All	1 221	12.21	100.00
C--	31	0.31	2.54
C-+	560	5.60	45.86
C+-	565	5.65	46.27
C++	65	0.65	5.32

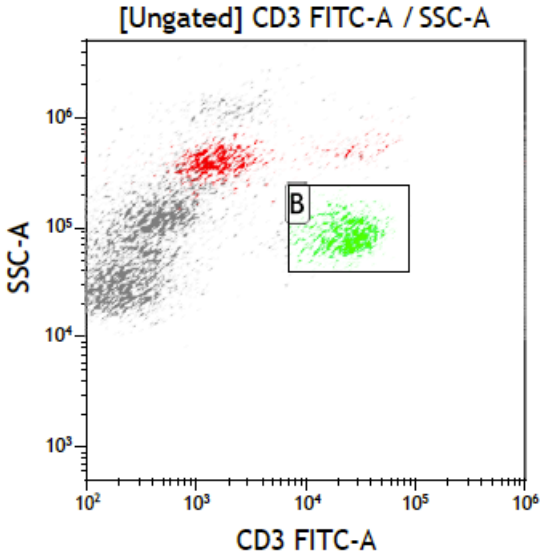
MNL sub-population 4



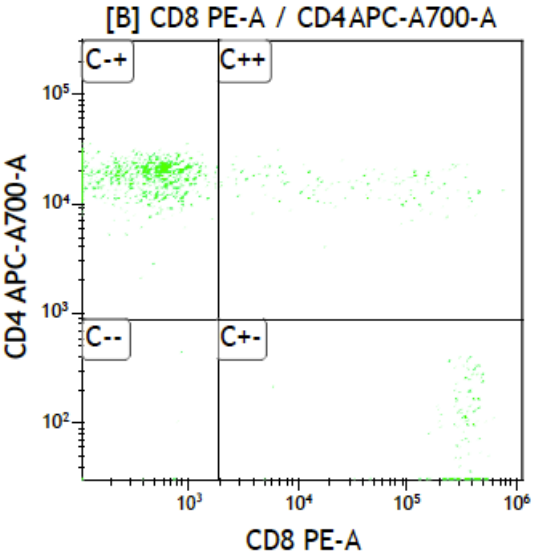
Gate	Number	%Total	%Gated
All	10 000	100.00	100.00



Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
A	1 125	11.25	11.25



Gate	Number	%Total	%Gated
All	10 000	100.00	100.00
B	1 265	12.65	12.65



Gate	Number	%Total	%Gated
All	1 265	12.65	100.00
C--	6	0.06	0.47
C-+	1 002	10.02	79.21
C+-	124	1.24	9.80
C++	133	1.33	10.51

Sheet 5: Raw data obtained for MNL cells with multiplex suspension array assay

Type	Description	Group	Cell type	TB strain	JG7 conc.	LPS	IL-1β	IL-6	IL-8	IL-10	IL-12p70	IFN-γ	TNF-α
X1	05/12 PBMC Cont 1	1	MNL			0	216.67	6103.17	9070.13	545.18	9.06	OOR <	294.56
X2	PBMC EC 1	1	MNL	H37Rv		0	7993.62	*28131.13	11790.07	2103.33	97.91	3487.92	16922.99
X3	PBMC E 1	1	MNL	H37Rv	25 µg/mL	0	*8062.67	*27871.09	18618.4	1977.84	58.82	1279.04	14598.05
X4	PBMC LPS 1	1	MNL			1	4831.16	*27858.59	10376.12	4585.76	54.19	OOR <	3211.8
X5	05/12 PBMC Cont 2	1	MNL			0	138.47	3089.54	8095.8	485.53	38.76	OOR <	214.95
X6	PBMC EC 2	1	MNL	H37Rv		0	7915.42	*27906.35	10750.97	1847	117.7	2869.92	16097.84
X7	PBMC E 2	1	MNL	H37Rv	25 µg/mL	0	8006.98	*27088.94	12721.29	2292.02	58.82	1329.36	14875.35
X8	PBMC LPS 2	1	MNL			1	4591.92	*27534.92	12473.35	4810.14	58.82	OOR <	3156.24
X9	09/12 PBMC Cont 1	2	MNL			0	116.6	1481.89	10044.63	329.4	38.76	OOR <	249.68
X10	PBMC EC 1	2	MNL	H37Rv		0	7639.22	*26053.68	11100.09	1893.75	135.87	2337.22	13092.34
X11	PBMC E 1	2	MNL	H37Rv	25 µg/mL	0	8000.61	*26253.67	11845.84	2946.32	38.76	2412.14	10579.48
X12	PBMC LPS 1	2	MNL			1	2913.99	23345.81	13314.38	4646.5	58.82	OOR <	1885.98
X13	09/12 PBMC Cont 2	2	MNL			0	74.29	827.8	7157.35	238.79	26.35	OOR <	202.8
X14	PBMC EC 2	2	MNL	H37Rv		0	7595.46	23994.88	11991.96	1937	141.64	2485.99	13655.32
X15	PBMC E 2	2	MNL	H37Rv	25 µg/mL	0	*8129.37	*27447.11	13170.43	3083.12	49.34	2630.72	11015.14
X16	PBMC LPS 2	2	MNL			1	2804.63	22991.03	13905.16	4588.87	58.82	OOR <	1746.08
X17	10/12 PBMC Cont 1	3	MNL			0	177.48	4976.72	8434.69	435.17	9.06	OOR <	365.34
X18	PBMC EC 1	3	MNL	H37Rv		0	7550.91	*27541.68	8858.29	2716.09	83.47	15893.27	16966.15
X19	PBMC E 1	3	MNL	H37Rv	25 µg/mL	0	7876.74	*28456.29	11177.1	3360	26.35	9910.47	20480.15
X20	PBMC LPS 1	3	MNL			1	3110.62	*27659.09	9171.7	5492.66	38.76	OOR <	3366.2
X21	10/12 PBMC Cont 2	3	MNL			0	196.7	5599.59	7644.49	411.02	26.35	OOR <	408.24
X22	PBMC EC 2	3	MNL	H37Rv		0	7482.49	*27862.00	8575.53	2215.79	58.82	8859.25	15152.95
X23	PBMC E 2	3	MNL	H37Rv	25 µg/mL	0	7593.1	*27622.93	9596.86	3300.06	26.35	10246.1	16537.66
X24	PBMC LPS 2	3	MNL			1	1744.47	*25541.26	9036.02	3401.16	38.76	OOR <	1838.33
X25	11/12 PBMC Cont 1	4	MNL			0	1023.07	6630.47	9183.39	312.09	49.34	OOR <	1619.79
X26	PBMC EC 1	4	MNL	H37Rv		0	7932.35	*28434.38	12401.95	1856.09	380.56	6432.98	31454.96
X27	PBMC E 1	4	MNL	H37Rv	25 µg/mL	0	7422.95	*27534.92	10187.25	2861.8	75.73	1139.48	23077.05
X28	PBMC LPS 1	4	MNL			1	7342.21	*27991.77	10731.54	5544.15	141.64	127.04	8181.42
X29	11/12 PBMC Cont 2	4	MNL			0	615.06	3699.82	8784.83	249.13	49.34	OOR <	1008.29
X30	PBMC EC 2	4	MNL	H37Rv		0	7547.99	*28174.64	8666.41	1763.62	258.85	3302.05	26384.39
X31	PBMC E 2	4	MNL	H37Rv	25 µg/mL	0	*8049.18	*27849.50	12223.09	2899.74	75.73	1056.93	23809.34
X32	PBMC LPS 2	4	MNL			1	7364.66	*28119.69	8077.53	5170.9	144.48	252.25	7862.93

Description: This table presents the cytokine data obtained for human MNLs following interaction with *M. tuberculosis* strain H37Rv, mAb JG7, and LPS, after 24 hours of incubation. Numbers represent observed concentrations (in pg/mL) for each cytokine, *Value = Value extrapolated beyond standard range, OOR = Out of Range, OOR< = Out of Range (Below range).

Appendix E: Ethics approval certificate

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Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

13/09/2018

Approval Certificate
New Application

Ethics Reference No: 487/2018

Title: Assessment of macrophage and granulocyte cell-enhanced phagocytosis of Mycobacterium tuberculosis by novel monoclonal antibodies

Dear Miss BongAkee Shey

The **Amendment** as described in your documents specified in your cover letter dated 30/08/2018 received on 30/08/2018 was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 12/09/2018.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**487/2018**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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